CRANFIELD UNIVERSITY

NATASHA SAHGAL

# MICROBIAL AND NON-MICROBIAL VOLATILE FINGERPRINTS: POTENTIAL CLINICAL APPLICATIONS OF ELECTRONIC NOSE FOR EARLY DIAGNOSES AND DETECTION OF DISEASES

CRANFIELD HEALTH

PhD THESIS

CRANFIELD UNIVERSITY

CRANFIELD HEALTH

PhD THESIS

ACADEMIC YEAR 2007-2008

NATASHA SAHGAL

# MICROBIAL AND NON-MICROBIAL VOLATILE FINGERPRINTS: POTENTIAL CLINICAL APPLICATIONS OF ELECTRONIC NOSE FOR EARLY DIAGNOSES AND DETECTION OF DISEASES

SUPERVISOR: PROF. NARESH MAGAN

JANUARY 2008

This thesis is submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy

# Abstract

This is the first study to explore the potential applications of using qualitative volatile fingerprints (electronic nose) for early detection and diagnosis of diseases such as dermatophytosis, ventilator associated pneumonia and upper gastrointestinal cancer. The investigations included *in vitro* analysis of various dermatophyte species and strains, antifungal screening, bacterial cultures and associated clinical specimens and oesophageal cell lines. Mass spectrometric analyses were attempted to identify possible markers.

The studies that involved e-nose comparisons indicated that the conducting polymer system was unable to differentiate between any of the treatments over the experimental period (120 hours). Metal oxide-based sensor arrays were better suited and differentiated between four dermatophyte species within 96 hours of growth using principal component analysis and cluster analysis (Euclidean distance and Ward's linkage) based on their volatile profile patterns. Studies on the sensitivity of detection showed that for *Trichophyton mentagrophytes* and *T. rubrum* it was possible to differentiate between log3, log5 and log7 inoculum levels within 96 hours. The probabilistic neural network model had a high prediction accuracy of 88 to 96% depending on the number of sensors used.

Temporal volatile production patterns studied at a species level for a *Microsporum* species, two *Trichophyton* species and at a strain level for the two *Trichophyton* species; showed possible discrimination between the species from controls after 120 hours. The predictive neural network model misclassified only one sample. Data analysis also indicated probable differentiation between the strains of *T. rubrum* while strains of *T. mentagrophytes* clustered together showing good similarity between them.

Antifungal treatments with itraconazole on *T. mentagrophytes* and *T. rubrum* showed that the e-nose could differentiate between untreated fungal species from the treated

fungal species at both temperatures (25 and 30°C). However, the different antifungal concentrations of 50% fungal inhibition and 2 ppm could not be separated from each other or the controls based on their volatiles.

Headspace analysis of bacterial cultures *in vitro* indicated that the e-nose could differentiate between the microbial species and controls in 83% of samples (n=98) based on a four group model (gram-positive, gram-negative, fungi and no growth). Volatile fingerprint analysis of the bronchoalveolar lavage fluid accurately separated growth and no growth in 81% of samples (n=52); however only 63% classification accuracy was achieved with a four group model. 12/31 samples were classified as infected by the e-nose but had no microbiological growth, further analysis suggested that the traditional clinical pulmonary infection score (CPIS) system correlated with the e-nose prediction of infection in 68% of samples (n=31).

No clear distinction was observed between various human cell lines (oesophageal and colorectal) based on volatile fingerprints within one to four hours of incubation, although they were clearly separate from the blank media. However, after 24 hours one of the cell lines could be clearly differentiated from the others and the controls. The different gastrointestinal pathologies (forming the clinical samples) did not show any specific pattern and thus could not be distinguished.

Mass spectrometric analysis did not detect distinct markers within the fungal and cell line samples, but potential identifiers in the fungal species such as 3-Octanone, 1-Octen-3-ol and methoxybenzene including high concentration of ammonia, the latter mostly in *T. mentagrophytes,* followed by *T. rubrum* and *Microsporum canis,* were found.

These detailed studies suggest that the approach of qualitative volatile fingerprinting shows promise for use in clinical settings, enabling rapid detection/diagnoses of diseases thus eventually reducing the time to treatment significantly.

# Acknowledgements

I would like to specially thank my supervisor, Prof. Naresh Magan for his continuous encouragement, advice and help during the course of my research.

Next, I express my thanks to Dr. Dave Aldred and Dr. Conrad Bessant for their patience, time and guidance through certain lab procedures and computational models. Special thanks to Dr. Claire Turner for all her help, technical support and advice with the MS analyses.

I am also grateful to Lee Humphreys and Dr. Catherine Kendall from the Gloucester Hospital NHS Trust for providing me the clinical samples including their help in the understanding and analysis of the clinical data.

I also express my gratitude to Prof. Hugh Barr from the Gloucester Hospital NHS Trust and Dr. Barry Monk and Mohammad Wasil from the Bedford Hospital NHS Trust for funding and supporting this project.

A very special thank you to my Father and Anuj, a dear friend, for being there constantly and offering their support, encouragement and understanding throughout, which has helped me keep my composure.

Finally, to all my colleagues in the lab and office who have been there whilst I was feeling lost.

# Table of contents

# List of Figures

# List of Tables and Plates

# Abbreviations

| | |
|---|---|
| AI | Artificial Intelligence |
| AIDS | Acquired Immune Deficiency Syndrome |
| AMDIS | Automated Mass Spectral Deconvolution and Identification System |
| ANN | Artificial Neural Network |
| AP-PCR | Arbitrarily primed polymerase chain reaction |
| ATCC | American Type Culture Collection |
| ATD | Automated Thermal Desorption |
| BAL | Bronchoalveolar Lavage |
| BBS | Blind Bronchial Sampling |
| BHI | Brain Heart Infusion |
| CA | Cluster Analysis |
| CAP | Community Associated Pneumonia |
| CFU | Colony Forming Unit |
| CHO | Chinese Hamster Ovary |
| C-NS | Coagulase Negative *Staphylococci* |
| CP | Conducting Polymer |
| CPIS | Clinical Pulmonary Infection Score |
| CSF | Cerebrospinal Fluid |
| csPCNA | Cancer Specific Proliferating Cell Nuclear Antigen |
| CT | Computed Tomography |
| Da | Daltons |
| DFA | Discriminant Function Analysis |
| DLC | Discotic Liquid Crystals |
| DMEM | Dulbecco's Modified Eagle's Medium |

| | |
|---|---|
| DMSO | Dimethyl Sulfoxide |
| DNA | Deoxyribonucleic acid |
| EA | Endotracheal Aspirate |
| ECACC | European Collection of Cell Cultures |
| ENT | Ear Nose Throat |
| EUS | Endoscopic Ultrasonography |
| FCS | Foetal Calf (Bovine) Serum |
| FIA | Flow Injection Analysis |
| GC | Gas Chromatography |
| GI | Gastrointestinal |
| GP | General Practitioner |
| HAP | Hospital Acquired Pneumonia |
| HCAP | Health-care Associated Pneumonia |
| HCN | Hydrogen Cyanide |
| HLA | Human Leukocyte Antigen |
| HS-SPME | Headspace – Solid Phase Micro Extraction |
| ICU | Intensive Care Unit |
| ITZ | Itraconazole |
| KNN | K – Nearest Neighbour |
| LD | Lethal Dose |
| LDA | Linear Discriminant Analysis |
| MALDI-TOF | Matrix Assisted Laser Desorption/Ionisation – Time Of Flight |
| MEMS | Microelectromechanical System |
| MHC | Major Histocompatibitlity Complex |
| MHz | Mega Hertz |

| | |
|---|---|
| MLP | Multilayer Perceptron |
| MOS | Metal Oxide Semiconductor |
| MOSFET | Metal Oxide Semiconductor Field Effect Transistor |
| MP | Metalloporphyrins |
| MRI | Magnetic Resonance Imaging |
| MRSA | Methicillin resistant *Staphylococcus aureus* |
| MS | Mass Spectrometry |
| MSSA | Methicillin susceptible *Staphylococcus aureus* |
| MV | Mechanical Ventilation |
| NCCLS | National Committee on Clinical Laboratory Standards |
| NCPF | National Collection of Pathogenic Fungi |
| NIST | National Institute of Standards and Technology |
| PARC | Pattern recognition |
| PBS | Phosphate Buffered Saline |
| PC | Principal Components |
| PCA | Principal Component Analysis |
| PCR | Polymerase Chain Reaction |
| PLS | Partial Least Squares |
| PNN | Probabilistic Neural Network |
| PSB | Protected Specimen Brush |
| PTFE | Polytetrafluoroethylene |
| QCM | Quartz Crystal Microbalance |
| QMB | Quartz Microbalance |
| RAPD | Random Amplification of Polymorphic DNA |
| RBF | Radial Basis Function |

| | |
|---|---|
| RE | Restriction Enzyme |
| RFLP | Restriction Fragment Length Polymorphism |
| RO | Reverse Osmosis |
| RPMI | Roswell Park Memorial Institute |
| SABHI | Sabouraud Brain Heart Infusion |
| SAW | Surface Acoustic Wave |
| SD | Sabouraud Dextrose |
| SDA | Sabouraud Dextrose Agar |
| SELDI-TOF | Surface-Enhanced Laser Desorption/Ionization – Time Of Flight |
| SHA | Static Headspace Analysis |
| SIMCA | Soft Independent Modelling of Class Analogy |
| SIFT-MS | Selected Ion Flow Tube – Mass Spectrometry |
| SOM | Self – Organising Map |
| SPR | Surface Plasmon Resonance |
| TIC | Total Ion Chromatogram |
| UV | Ultraviolet |
| VAP | Ventilator Associated Pneumonia |
| VOC | Volatile Organic Compound |

# *Chapter 1*

# *LITERATURE REVIEW*

## *1.1 Introduction*

"Prevention is better than cure" is a well known idiom and is something that every individual in the present day desires. From the youth to the aged, people have become very conscious of the attributes of good health and therefore averse to any illness. Preventive medicine, in today's world, is thus fast becoming an accepted therapeutic approach. It not only prevents the onset of a disease but also decreases the time spent by GPs on patients and the overall money spent on medical treatment. Patients too are beginning to demand rapid and early diagnosis of diseases, be they of microbial or non-microbial origins. Moreover, early differentiation between different infections is an important factor that facilitates rapid treatment as part of a preventative health strategy (Turner & Magan, 2004).

A good example that indicates preventive medicine can work wonders is the use of vaccines against diseases such as hepatitis, polio or measles. However, over the years a number of diseases have emerged and are constantly increasing due to growing microbial resistance or genetic or environmental causes. For such diseases, we need to have good diagnostic tools that detect them at an early stage with high sensitivity and specificity. Recent advances in technology have resulted in a big leap in medical diagnostics with devices such as CT scans or magnetic resonance imaging. These procedures are nevertheless expensive and require highly trained personnel for their operation due to the nature of the equipment.

Therefore, the current research focus is on trying to find/develop diagnostic techniques that not only detects the disease in its infancy but is also economical and easy to operate.

## *1.2 Dermatophytosis*

There has been a dramatic increase in the incidence of mycotic infections like dermatophytoses over the past decade due to heightened susceptibility in the immune system of populations. These include patients with AIDS, on immunosuppressive therapy, i.e. chemotherapy, and undergoing more invasive diagnostic and surgical procedures (prosthetic implants). Due to the fact that any fungus may incite an infectious disease in a host with altered immunity, organisms that were once harmless now have the potential to be pathogenic (Rinaldi, 2000; Di Salvo, 2005).

Dermatophytes are responsible for one of the most common human fungal infectious diseases in the world and are the leading cause of hair, nail and skin infections in humans. They are a group of morphologically and physiologically related moulds that cause a well-defined infection – dermatophytosis, also known as ringworm or tinea and are keratinophilic and keratinolytic in nature. This implies that they possess the ability to digest keratin *in vitro* and utilise it as a substrate and some may invade tissues (such as hair, nails and skin) *in vivo* and provoke tineas (Simpanya, 2000). Infection is usually restricted to the nonliving cornified layers and is cutaneous, where the fungus only colonises but does not invade the living tissue. However, the metabolic products can cause allergic and inflammatory responses in the host (Weitzman & Summerbell, 1995). The breakdown of the keratinised cells is due to the enzyme keratinase produced by these organisms. In very rare instances these fungi penetrate the deeper tissues or organs in immunocompromised hosts and develop into granulomas (Hiruma & Yamaguchi, 2003).

### 1.2.1 Aetiology, ecology and epidemiology

These fungi are classified into three genera namely *Epidermophyton, Microsporum* and *Trichophyton* and vary not only according to the anatomic location of infection but also their geographical occurrence – widespread distribution or regional restriction. Furthermore, these are also divided into three groups based on their natural habitat: (1) Geophilic species – primarily live in soils, associated with breakdown of fallen keratinous materials i.e. hair, feathers, hooves and horns and may cause severe reactions on interaction with humans or animals e.g. *M. gypseum*; (2) Zoophilic species – generally infects animals but can cause occasional human infections e.g. *M. canis* and finally (3) Anthropophilic species – primarily human pathogens rarely infecting animals e.g. *E. floccosum*. These anthropophilic fungi have the strongest infective ability and are known to possess polymorphous morphological variations (Weitzman & Summerbell, 1995; Hiruma & Yamaguchi, 2003). Table 1.1 shows the prevalence and geographical distribution of the human pathogenic species used in this study as adapted from Hiruma & Yamaguchi (2003).

Epidemiology plays an important role in controlling infections and identifying the source and route of infections. However, due to the widespread nature of certain common dermatophyte species it has been difficult to do so. The general routes of contracting infection are either by direct contact with infected individuals and/or animals or by indirect transmission via infected materials or fomites. The latter can account for common usage of items such as combs, towels, footwear, clothing and linen; sharing communal facilities like swimming pools, showers, public baths and gymnasium; contact sports and damp foot conditions. An interesting feature of these fungal species is that their infectious spores persist in the environment for a long time

thus enabling the possibility of re-infection (Weitzman & Summerbell, 1995; Hiruma & Yamaguchi, 2003). A recent study showed that the socioeconomic status, living in dormitories and poor hygiene and sanitary conditions increased the incidence of dermatophytoses in children (Metintas *et al.*, 2004).

**Table 1.1:** Ecology and prevalence of dermatophyte species used in the current study, adapted from Hiruma & Yamaguchi (2003).

| Species | Origin | Geographic distribution | Prevalence |
|---|---|---|---|
| *T. mentagrophytes* | | | |
| *var. mentagrophytes* | Zoophilic (Rodent) | Worldwide | Common |
| *var. interdigitale* | Anthropophilic | Worldwide | Common |
| *var. erinacei* | Zoophilic (Hedgehog) | Europe, New Zealand, Africa | Occasional |
| *var. quinckeanum* | Zoophilic (Mouse) | Worldwide | Rare |
| *T. rubrum* | Anthropophilic | Worldwide | Common |
| *T. verrucosum* | Zoophilic (Cow) | Worldwide | Common |
| *T. violaceum* | Anthropophilic | Europe, Africa, Asia | Common |
| *M. canis* | Zoophilic (Cat, Dog) | Worldwide | Common |
| *var. distortum* | Zoophilic (Cat, Dog) | New Zealand, USA | Rare |

### 1.2.2 Clinical manifestations

A wide range of clinical features have been presented by dermatophytoses, which are influenced by factors such as the species, size of inoculum and the host's immune status but mainly depends on the site of infection. Instead of a single organism causing one sign of the disease, several species can, in fact, result in a single disease manifestation (Hiruma & Yamaguchi, 2003). The various clinical features (Figure 1.1) are as follows:

a) Tinea Corporis: Ringworm of the body usually involving the trunk, shoulders or limbs and can be caused by any dermatophyte.

b) Tinea Pedis (Athlete's foot): Ringworm of the feet especially the soles and presents as scaling or macerations between toes. It is frequently caused by *T. rubrum* and *T. mentagrophytes.*

c) Tinea Crusis (Jock itch): Ringworm of the groin with occasional infection of upper thighs and usually seen in men. Frequent etiologic agents are *T. rubrum* and *E. floccosum.*

d) Tinea Capitis: Ringworm of the scalp, where spores are formed within the hair shaft – endothrix infection or outside it – ectothrix infection and caused by *Microsporum* or *Trichophyton* species. Common paediatric disease with increasing incidence in UK especially within the Afro-Caribbean population (Fuller *et al.*, 2003).

e) Tinea Favosa: Severe, chronic infection of the scalp in humans with crust formation around hair shafts. It is caused by *T. schoenleinii* and is common in Eurasia and Africa.

f) Tinea Barbae: Infection of the bearded area caused by *T. mentagrophytes* and *T. verrucosum.*

g) Tinea Manuum: Infection of the palms, mainly caused by *T. rubrum.*

h) Tinea Unguium: Invasion of the nail plate, usually in toenails. Commonly caused by *T. mentagrophytes* and *T. rubrum.* Also known as onychomycosis.

i) Tinea Imbricata: Chronic infection on the body characterised by concentric rings and caused by *T. concentricum.* It is geographically restricted to Southeast Asia, Mexico and Central and South America (Weitzman & Summerbell, 1995; Hay, 2003).

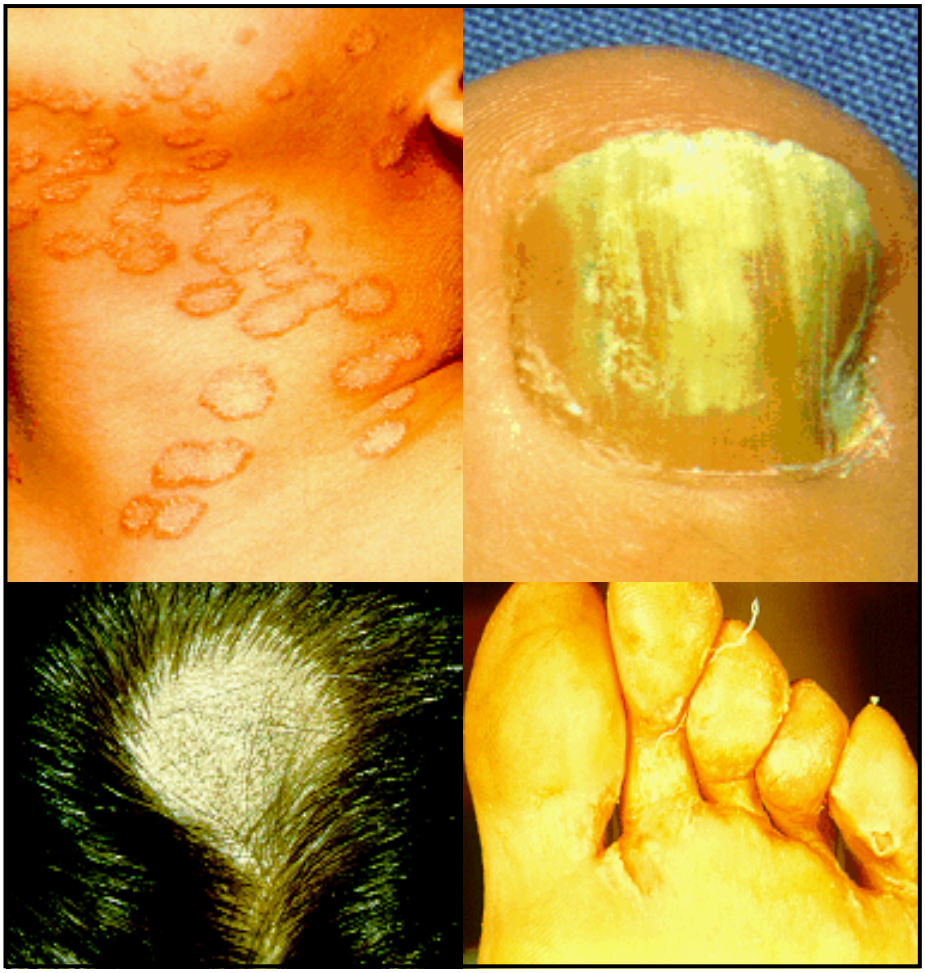


**Figure 1.1:** Images depicting tinea corporis, tinea unguium, tinea pedis and tinea capitis in clockwise direction from the top left corner (Ellis, 2007).

### 1.2.3 Methods of identification and differentiation

Conventional laboratory procedures for the identification of dermatophytes rely on microscopic examination – determining morphological structures such as conidia and hyphae; *in vitro* culture which is based on colony pigmentation, texture and growth rate and biochemical tests such as the urease test and bromocresol purple-milk solids-glucose medium (Weitzman & Summerbell, 1995). However, these are time consuming, expensive, lack specificity and require specialist skills. Thus, indicating a need for rapid and improved diagnostic procedures.

Advances in the molecular diagnostics sector have led to the development of numerous approaches using nucleic acid amplification and restriction enzyme (RE) techniques for the rapid identification of dermatophytes as well as strain typing of the commonly occurring species. Studies with different random primers using the arbitrarily primed – polymerase chain reaction (AP-PCR) method have differentiated between *Microsporum* or *Trichophyton* genera as well as some selected species except a few of the latter. The DNA fragments produced characteristic band patterns enabling distinction, although the two genera were found to be genetically similar (Liu *et al.*, 1996; Liu *et al.*, 1997; Liu *et al.*, 2000). Using a combination of primers, however, increased the discrimination between species except for *T. rubrum* and *T. gourvillii* (Liu *et al.*, 2000).

The dermatophytes were also shown to be clearly distinct from other fungal and yeast species such as *Scytalidium, Fusarium, Aspergillus* and *Candida* using AP-PCR and PCR – restriction fragment length polymorphism (PCR-RFLP) (Liu *et al.*, 2000; Machouart-Dubach *et al.*, 2001). Although in the latter the dermatophyte species could not be differentiated from each other.

Restriction enzyme analysis in conjunction with PCR has been applied for species identification making use of genomic as well as ribosomal DNA sequences (Jackson *et al.*, 1999; Shin *et al.*, 2003; Kamiya *et al.*, 2004). These studies made use of different species with a few common ones such as *T. mentagrophytes, T. rubrum, M. gypseum, M. canis* and *E. floccosum*, but in case of the first two studies at least two species within the *Microsporum* and *Trichophyton* genera respectively were found to be indistinguishable based on the enzyme digestion patterns.

Turin *et al.* (2000) reported the use of three primer pairs in a PCR assay that facilitated the recognition of pathogenic fungi in clinical samples. Another study employed PCR fingerprinting to identify common dermatophyte species where species specific profiles were produced. However, it is costlier than the conventional methods (Faggi *et al.*, 2001). Molecular analyses of the chitin synthase 1 gene along with phylogenetic analysis were used to identify a clinical isolate from a black-dot ringworm lesion (Okabayashi *et al.*, 1999).

Recently, Hollemeyer *et al.* (2005) presented a novel application using matrix assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry which makes use of the mass spectrum of cleaved peptides to distinguish between fungal and non-fungal infections. It allowed clear differentiation of *T. rubrum* infections from those with psoriasis and healthy people. Another recent method involves the use of real time PCR assays for differentiation of dermatophyte species (Arabatzis *et al.*, 2007).

On the other hand, there have been slightly varying/ambiguous reports pertaining to strain identification of the two common *Trichophyton* species. Some reported either no or very little differences between strains of *T. rubrum* (Liu *et al.*, 1996; Zhong *et al.*, 1997; Gräser *et al.*, 1999) whilst others demonstrated the presence of molecular diversity using clinical isolates (Jackson *et al.*, 1999; Jackson *et al.*, 2000; Kamiya *et al.*, 2004; Baeza & Giannini, 2004; Baeza *et al.*, 2006) based on different molecular methods. Baeza *et al.* (2006) also showed that epidemiologically related strains of this species possessed a high degree of similarity.

Similarly, intraspecific variability was found to be present in *T. mentagrophytes* strains using RAPD and PCR fingerprinting respectively (Kac *et al.*, 1999; Faggi *et al.*, 2001) but in contrast Liu *et al.* (1996) found no differences in the strains of this species using AP-PCR. Moreover, two recent studies have shown to reduce the time for identification of dermatophytes especially that of *T. rubrum* using PCR to either 5 or 48 hours respectively (Kardjeva *et al.*, 2006; Brillowska-Dąbrowska *et al.*, 2007).

Most of these PCR methods can be expensive, suffer from either DNA contamination or potential enzymatic inhibitors (Binstock, 2007) and thus not feasible for clinical analyses. The current study therefore focuses on a novel method based on volatile production patterns to identify dermatophytes, especially the *Trichophyton* species and determine any possible inter-strain differences.

### 1.2.4 Antifungal susceptibility

Unlike some of the other diseases, dermatophytosis is not life threatening. Nevertheless, in order to initiate/facilitate appropriate treatment it is essential to determine the causative organism down to the species level. The importance of accurate diagnosis is further substantiated by the expenses of drugs, longer therapy especially in case of nail and scalp infections and certain associated risks including antibiotic resistance. Treatment regimens can either be topical or systemic (oral) depending on the type and severity of infection (Gupta & Tu, 2006).

Antifungal susceptibility tests are known to help facilitate screening, in guiding the selection of drugs and to isolate the presence of resistant strains. However, no standard clinical method exists, therefore many susceptibility studies have been performed to

determine the *in vitro* activity of antifungal agents, common ones being terbinafine, itraconazole, fluconazole and griseofulvin, against the dermatophytes in an attempt to develop a standard assay. The methods used range from broth microdilution assays to disk diffusion methods including the use of commercial systems (Fernandez-Torres *et al.*, 2002; Favre *et al.*, 2003; Esteban *et al.*, 2005; Santos *et al.*, 2006).

Over the recent years, there have been many studies on susceptibility involving alternative remedies using natural resources such as plant extracts or essentials oils for these infections owing to the side effects and increasing ineffectiveness of current medications. The use of extracts from various plants have been shown to inhibit growth of certain dermatophytic species by almost 80-100% *in vitro* with minimum inhibitory concentration levels comparable to those of currently used antifungals (Ali-Shtayeh & Abu Ghdeib, 1999; Gurgel *et al.*, 2005; Koc *et al.*, 2005; Silva *et al.*, 2005). Moreover, essential oils were shown to have a synergistic effect when combined with an antifungal agent (Shin & Lim, 2004). Another study using aqueous onion extracts also showed molecular changes such as formation of resistant forms in *T. rubrum* apart from its inhibitory effects on the two fungal species (Ghahfarokhi *et al.*, 2004).

The studies undertaken so far are time consuming and can be difficult to interpret mainly due to the lack of standard protocols. Hence, in this study the approach of volatile fingerprinting has been adopted to determine its feasibility for screening the dermatophytes against antifungals.

## *1.3 Ventilator associated pneumonia*

Infections of the lower respiratory tract normally tend to be serious, vary in severity and are one of the main causes of worldwide mortality pertaining to infectious diseases. One such example is pneumonia, classified based on the location of occurrence into either community-associated pneumonia (CAP) or health-care-associated pneumonia (HCAP) or hospital acquired pneumonia (HAP). The last being a major health concern although there have been advances in diagnostic procedures, therapeutic agents and supportive care (McEachern & Campbell, 1998; Kollef, 2005)

Nosocomial pneumonia i.e. HAP - an infection of the lungs caused by bacteria, fungi or viruses is the second most common hospital acquired infection after urinary tract infection. It has increased incidence in critically ill individuals (≈ 27%) and is responsible for the greatest number of nosocomial deaths. Annually the US reports about 300,000 such cases, accounting for 25% of intensive care unit (ICU) infections (McEachern & Campbell, 1998; Kollef, 2005; Flanders *et al.*, 2006). One of the most serious nosocomial infections that occur more than 48 hours after endotracheal intubation followed by mechanical ventilation (MV) is termed as ventilator associated pneumonia (VAP). It has frequent occurrence within the ICU, accurate diagnosis is difficult, lengthens hospital stay and is linked to increased mortality and morbidity (Chastre & Fagon, 2002; Hunter, 2006).

### 1.3.1 Aetiology and epidemiology

The microbial agents responsible for ventilator associated pneumonia vary according to the patient population, duration of stay in the hospital or ICU, the diagnostic procedures and the time of onset of the disease. Based on the time of onset, VAP is classified as

either early-onset VAP – that occurs within the first four days of mechanical ventilation or late-onset VAP – that develops five days or more after initiation of mechanical ventilation (Craven, 2000; Kollef, 2005). Not only is the former less severe in form with better prognosis but also caused by antibiotic sensitive bacteria. The latter however occurs more likely due to antibiotic resistant bacteria (Kollef, 2005).

Mostly, the occurrence of VAP in individuals has been known to be polymicrobial, especially due to aerobic gram negative bacilli and also increasingly due to gram positive bacteria. Those responsible for early-onset VAP are *Streptococcus pneumoniae*, *Haemophilus influenzae*, methicillin-sensitive *Staphylococcus aureus* (MSSA) or normal endogenous flora whilst those causing late-onset VAP are *Pseudomonas aeruginosa*, *Acinetobacter* species, Enterobacteriaceae species or methicillin-resistant *S. aureus* (MRSA) (Koeman *et al.*, 2001; Chastre & Fagon, 2002). Other notable features that can affect the aetiology of the disease are age, prior usage of antibiotics or broad spectrum drugs, mechanical ventilation for at least seven days before the onset of VAP and certain underlying diseases (e.g. *H. influenzae, S. pneumoniae* increases risk for chronic lung disease while *S. aureus* for trauma, neurologic and diabetic patients) (Cavalcanti *et al.*, 2005).

The National Nosocomial Infection Surveillance System indicated that in the US pneumonia accounts for one third of all nosocomial infections, 83% of which are due to mechanical ventilation (Shorr & Kollef, 2005). Thus, based on various European and American studies the overall incidence of VAP varies from 8 to 28% (Chastre & Fagon, 2002; Rello *et al.*, 2002; Cavalcanti *et al.*, 2005; Hunter, 2006). In one study, the incremental risk of pneumonia was shown to be 1% per day; contrarily another study

showed that although the cumulative risk of acquiring VAP increased over time, the daily hazard rate declined after day 5 (Cook *et al.*, 1998; Chastre & Fagon, 2002).

Apart from increased incidence, VAP is also the leading cause of nosocomial mortality and morbidity and is associated with excessive costs especially for patients in the intensive care units. Its mortality rate can range from 24 to 50%, but can also reach 76% in specific settings such as underlying disease, severity of host response or when lung infections are caused by high risk pathogens, namely gram negative bacilli (Craven, 2000; Chastre & Fagon, 2002; Hunter, 2006). Consequently, this results in prolonged hospital stay, ultimately causing additional financial burden as the costs can increase by approximately $40,000 per patient (Rello *et al.*, 2002). These aspects tend to differ from country to country, hospital to hospital, health care systems as well as patient populations.

### 1.3.2 Pathogenesis and associated risk factors

The development of pneumonia implies the weakening of the host's immune responses due to microbial invasion of the normally sterile lower respiratory tract and lung parenchyma. This is caused by either a flaw in the host's defence mechanisms (such as anatomic barriers, cough reflexes, cell-mediated/humoral immunity or the phagocytic system), highly virulent micro-organisms or an extremely high inoculum (Cavalcanti *et al.*, 2005; Hunter, 2006).

According to Rumbak (2002) organisms can follow only four routes to infect the lower respiratory tract: inhalation, aspiration and haematogenous or contiguous spread. Aspiration being the primary route, especially microaspiration which is a two-step

process where initial bacterial colonisation of the oropharynx is followed by aspiration of the contaminated secretions (Kollef, 2005; Rumbak, 2005). Further, endotracheal intubation facilitates bacterial entry into the lung by suppressing the cough reflex thereby compromising the natural barrier. It also aids entry by pooling and leakage of contaminated secretions above or around the endotracheal cuff. These can further develop into a biofilm, which can disseminate the infectious material into the lungs by means of suctioning (McEachern & Campbell, 1998; Rumbak, 2002; Rumbak, 2005). Other potential sources include macroaspirations of gastric material, contaminated respiratory equipment, fibre-optic bronchoscopy, haematogenous spread from infected catheters or contiguously from the abdomen or paranasal sinuses or dental plaques as well as health care personnel (Chastre & Fagon, 2002; Rumbak, 2005). These numerous sources of infection can be seen as risk factors associated with disease development, which can help in providing both information regarding probability of infection and developing preventive measures. Figure 1.2 depicts the routes of infection as adapted from Morehead & Pinto (2000).

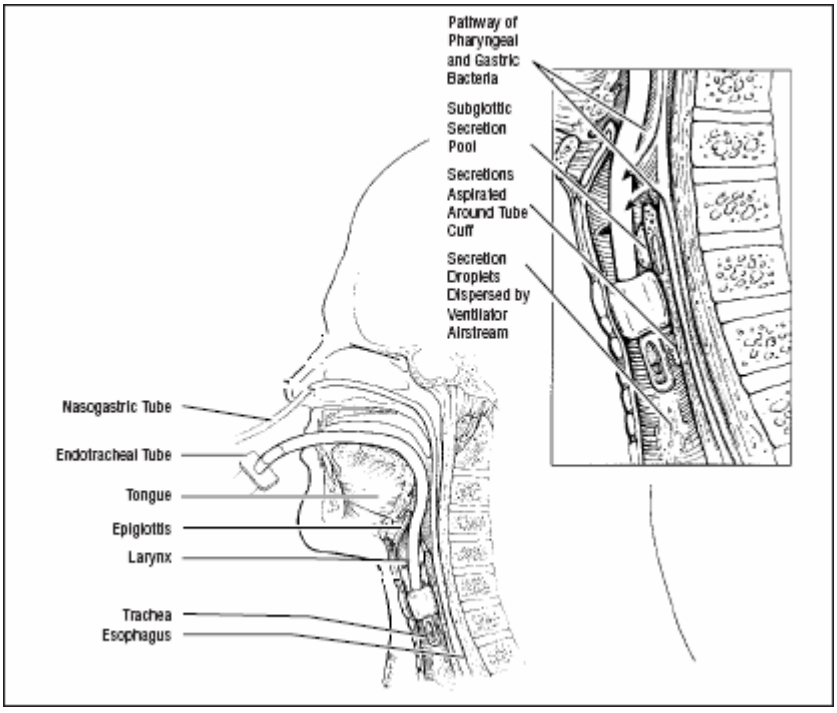



**Figure 1.2:** The pathogenic routes that cause ventilator associated pneumonia, adapted from Morehead & Pinto (2000).

### 1.3.3 Diagnosis of VAP

Despite there being several methods for diagnosing VAP, the absence of a 'gold standard' has fuelled controversy within this area. Moreover, these techniques have varying degrees of sensitivity and specificity which adds to speculation as to which techniques should be employed for appropriate diagnosis (Baughman, 2005a; Fujitani & Yu, 2006). Additionally diseases with similar clinical manifestations render diagnosing difficult. The methods available are based on clinical criteria and microbiological cultures using invasive or non-invasive procedures. The current diagnostic methods are summarised in Table 1.2 (Kirtland *et al.*, 1997; Chastre & Fagon, 2002; Pugin, 2002; Baughman, 2005b; Baughman, 2005a; Fagon & Chastre, 2005; Kollef, 2005; Flanders *et al.*, 2006; Hunter, 2006; Koenig & Truwit, 2006; Porzecanski & Bowton, 2006).

**Table 1.2:** Summary of the currently available procedures for the diagnosis of ventilator associated pneumonia (VAP).

| Diagnostic technique | Signs/Symptoms/Test | Specificity | Comments |
|---|---|---|---|
| *Clinical Evaluation* | ▪ Systemic signs of infection & inflammation | ▪ Non-specific | ▪ Finally, **CPIS score** developed based on signs |
| *(Starting point for suspected cases)* | ▪ New/worsening pulmonary infiltrates on chest radiographs | ▪ Non-specific | ▪ CPIS ≥ 6 ☞ VAP |
| | ▪ Increased/purulent tracheobronchial secretions | ▪ Non-specific | ▪ Clinical criteria alone not sufficient or specific |
| | ▪ Chest x-rays with air bronchograms | ▪ Indicative | |
| | ▪ Cultures/histopathology | ▪ Reports of failed findings have been observed | ▪ Disagreement between pathologists is common |
| *Bronchoscopy* | ▪ Bronchoalveolar lavage (BAL) } Invasive | ▪ More Sensitive | ▪ Thresholds $10^4$ & $10^3$ cfu $ml^{-1}$ respectively for BAL/PSB |
| *(Fibre-optic bronchoscopy)* | ▪ Protected specimen brushing (PSB) } Invasive | ▪ Less sensitive BUT high specificity | ▪ Threshold for pathogens is $10^5$-$10^6$ cfu $ml^{-1}$ |
| | | | ▪ Threshold for contaminants is $10^4$ cfu $ml^{-1}$ |
| *Non-bronchoscopy* | ▪ Cultures from Endotracheal aspirates (EA) | ▪ Highly sensitive BUT less specific | ▪ Threshold range of $10^5$ - $10^6$ cfu $ml^{-1}$ |
| | ▪ Blind sampling using BAL/PSB[§] | ▪ Improves specificity | ▪ [§]Not standardised |
| | ▪ Blind bronchial sampling (BBS)[§] | | ▪ [§]Use restricted to mechanically ventilated patients |

On comparison, bronchoscopic diagnosis helps in selecting antibiotic treatment, reduces its excessive use, and might help detect nonpulmonary infection. However, it is expensive and false positive or negative results are likely due to colonisation or contamination or prior antibiotic usage (Koenig & Truwit, 2006). Non-bronchoscopic methods in contrast are cheaper, less invasive and can be offered to patients with small endotracheal tubes. Additionally, they are available to nonbronchoscopists, prevent contamination of the proximal airway and decreases compromise on gas exchange. They are however prone to sampling errors inherent in a blind technique owing to no visualisation (Chastre & Fagon, 2002; Koenig & Truwit, 2006). Moreover quantitative cultures from these techniques can serve as a monitor to check the progress of antibiotic therapy.

### 1.3.4 Antimicrobial therapy: pros and cons

The emergence and spread of multi-drug resistant pathogens are a rising threat in hospitals and to the welfare of the patient. This is mainly attributed to the inappropriate use of, as well as, prolonged exposure to antibiotics (Hunter, 2006; Chastre & Luyt, 2007). These are also responsible for increasing mortality, hospital stay, costs, antibiotic toxicity and drug resistant organisms. Moreover the absence of a specific regimen for optimal treatment and duration emphasizes the need for appropriate initial antimicrobial therapy and to limit unnecessary antibiotic exposure (Fagon & Chastre, 2005; Kollef, 2005).

Antimicrobial de-escalation is an effective approach to achieve the above. Therefore, it is essential for prompt initiation of appropriate empiric antibiotics, which involves

starting with broad spectrum drugs or combination therapy* especially in case of high risk patients. Although rapid identification of causative pathogens is important, it should not delay prescription of antibiotics. Once the culture results are known the treatment can be narrowed (Kollef, 2003; Flanders *et al.*, 2006; Hunter, 2006). In order for this to succeed, clinicians need to be aware of their hospital antibiogram as well as the risk of other infections caused by the same pathogens (Kollef, 2003; Porzecanski & Bowton, 2006). On the other hand, to ensure unnecessary drug administration the duration of the treatment must be selected so as to prevent failure or relapse of infection. Chastre & Luyt (2007) showed that shorter 8 day regimes were not inferior to the 15 day duration ones except under certain circumstances. Studies have also shown that stopping antibiotics in patients with negative or lower BAL/PSB culture results and a clinical pulmonary infection score (CPIS) < 6 had no negative outcome on patients (Fagon & Chastre, 2005; Kollef, 2005; Shorr & Kollef, 2005; Solomkin, 2005; Porzecanski & Bowton, 2006).

## *1.4 Oesophageal malignancy*

Upper gastrointestinal (GI) malignancies include the oesophagus, stomach and pancreas where oesophageal cancer represents 7% of all GI cancers. It is one of the most lethal cancers and is the sixth leading cause of such deaths worldwide (Kumbasar, 2002). Over the last three decades there has been a dramatic increase in the incidence of oesophageal adenocarcinoma, considered unusual over oesophageal squamous cell carcinoma. It occurs more commonly in middle aged or elderly men, escalating in white males (Holmes & Vaughan, 2007).

*Combination therapy is used to provide adequate coverage for patients with prior drug exposure & prolonged MV.

Oesophageal cancer has the greatest geographical variation in incidence worldwide than any other cancer. High risk areas being Asia and parts of Africa rather than the western countries. However, alcohol consumption and smoking are the major risk factors that are increasing its occurrence in the western world, especially in UK. An estimated 7000 new cases were seen in the UK in 1997. In Asia, diet is also considered an important factor (Lamb & Griffin, 2003; Holmes & Vaughan, 2007).

### 1.4.1 The two common carcinomas

The two most common primary oesophageal malignancies are squamous cell carcinoma and adenocarcinoma. The former develops mainly within the upper and mid section of the oesophagus while the latter within the distal/lower end and the oesophagogastric junction. Adenocarcinomas mainly occur in the Barrett's oesophagus, an abnormality in the normal lining of the lower oesophageal wall-Barrett's metaplasia. Thus Barrett's oesophagus is known to be a precancerous condition seen in individuals with chronic gastric reflux disease (Merck Manual, 1999; Bateman, 2003). Furthermore, the differentiation between these two carcinomas can be difficult under certain conditions where appearances are similar (Kumbasar, 2002). In addition, it is also difficult to distinguish between adenocarcinomas of the oesophagus or that of the stomach invading the lower oesophagus (Merck Manual, 1999; Bateman, 2003). Figure 1.3 shows these different conditions.

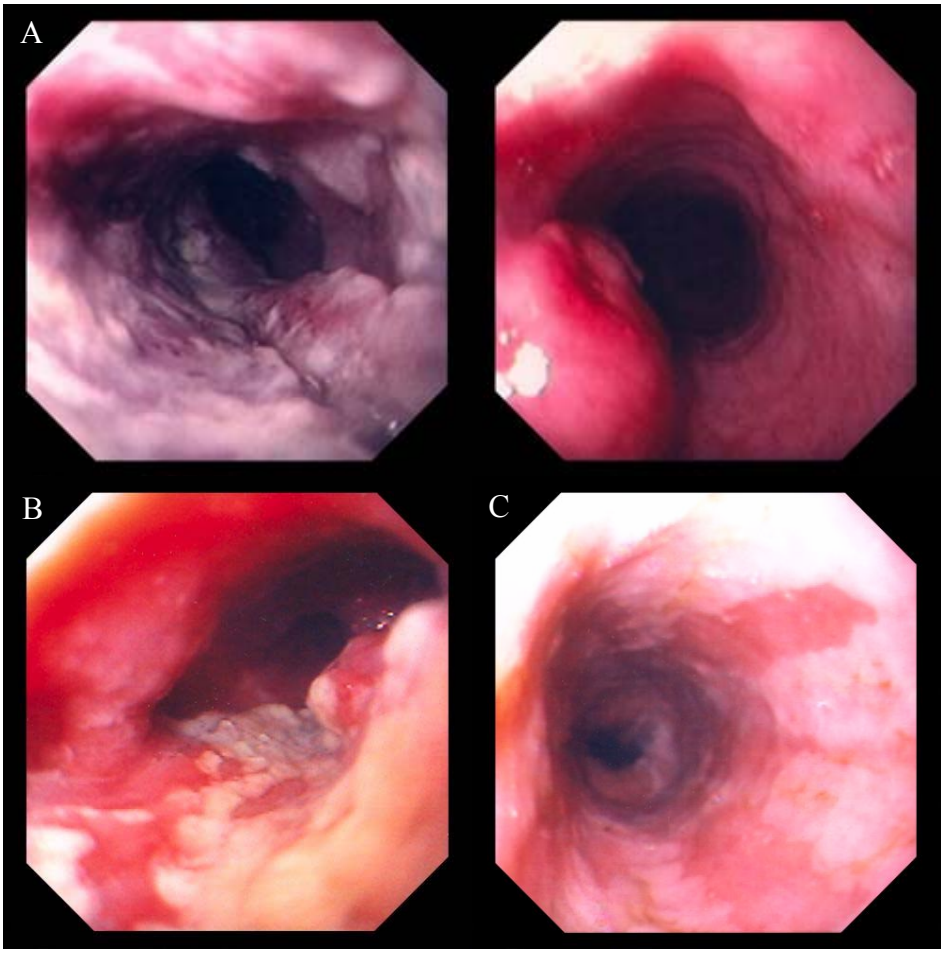



**Figure 1.3:** Endoscopic images depicting squamous cell carcinoma (A, upper panel), adenocarcinoma (B, left lower panel) and Barrett's oesophagus (C, right lower panel) adapted from the Merck manual (1999).

### 1.4.2 Current diagnostic tools

Oesophageal cancers are generally detected in their advanced stages because early stages tend to be asymptomatic and thus difficult to diagnose. Therefore, there is high risk of mortality and poor prognosis in such patients. Currently, the most favourable method adopted for diagnosing these carcinomas is upper GI endoscopy enabling collection of samples for tissue biopsy. Barium contrast radiography is also performed; yet it needs to be followed by endoscopy for biopsy (Lightdale, 1999).

The absence of screening procedures also hinders the possibility of early diagnosis. However, patients with Barrett's oesophagus are recommended routine endoscopic surveillance in order to check for adenocarcinoma, although its benefit remains a controversial issue. At the same time, Lugol's staining together with endoscopy has been used in order to detect squamous cell carcinoma early (Tachimori & Kato, 1998; Lightdale, 1999). These techniques are nevertheless invasive, unpleasant and carry the risk of perforation; hence there is need for alternative non-invasive diagnostic procedures, for instance breath analysis in patients for presence of specific volatile compounds.

The next step after diagnosis is to determine the stage of the tumour and extent of metastasis (staging) for which techniques such as computed tomography (CT) scan of the abdomen and chest, magnetic resonance imaging (MRI) and endoscopic ultrasonography (EUS) are used. The first two evaluate the extent of spread of the disease whilst the latter is better for detecting the depth of tumour invasion (Tachimori & Kato, 1998).

### 1.4.3 Markers for early diagnosis

The development of biomarkers for the detection of diseases is a popular aspect of research and increasing in importance in the diagnostic domain. Certain cancers, especially of the oesophagus are detected at advanced stages where not much can be done to avoid fatality. Therefore, there have been efforts for identifying markers for earlier diagnosis using molecular approaches and recently proteomic analyses.

Early studies have shown that elevated levels of p53 proteins in precancerous or cancerous oesophageal cells could serve as a potential marker (Wang *et al.*, 1993). Serological analyses by means of immunoassays in patients with cancer after routine diagnosis also suggest the possibility of serum p53 antibody to serve as a marker for early detection (Ralhan *et al.*, 2000; Shimada *et al.*, 2000). Other studies have identified certain DNA microsatellite markers that can be indicative of early squamous cell carcinoma. They also showed the cancer to be associated with genetic instability (Hu *et al.*, 2000; Lu *et al.*, 2003). Furthermore, serum microsatellite alterations in certain genes were regarded as suitable screening markers for patients with adenocarcinoma, as these alterations were absent in normal individuals (Eisenberger *et al.*, 2006).

Recent advances in proteomics especially using 2D differential gel electrophoresis with mass spectrometry have enabled identification of cancer specific protein markers. Examples being periplakin, a protein possible for early detection (Nishimori *et al.*, 2006) or tumour rejection antigen (gp96) found only in cancerous cells (Zhou *et al.*, 2002). Another recent study also demonstrated the potential of telomerase activity as a marker for oesophageal carcinoma in high risk populations using oesophageal balloon cytology as a relatively less invasive procedure (McGruder *et al.*, 2006). Most of the studies however, are invasive and expensive owing to endoscopy and have not yet found their way to clinics. Thus it is imperative to find non-invasive, clinically feasible procedures.

## *1.5 Volatile fingerprinting I: Chromatographic and mass spectrometric techniques*

Microbial species have been shown to produce a wide range of volatile organic compounds (VOCs) such as alcohols, ketones, aliphatic acids, terpenes and sulphur and nitrogen compounds, some of which possess characteristic odours. Studies using analytical techniques like gas chromatography (GC), GC linked to mass spectrometry (GC-MS) and lately headspace solid phase micro-extraction (HS-SPME) have been carried out to analyse the volatiles generated by micro-organisms (Turner & Magan, 2004). Factors influencing the amounts and patterns of volatile production include the type of microbial species, media for cultivation and the age of the culture (Schöller *et al.*, 1997; Turner & Magan, 2004).

However, GC-MS suffers from certain disadvantages such as need of adsorption traps for preconcentration of sample implying it is not an actual real-time monitoring device. It also requires knowledge of fragmentation patterns of each gas component, calibration for each trace gas for quantification and is impractical for detection of low molecular weight compounds, e.g. formaldehyde and ammonia. Due to these factors a new analytical technique has been developed for the real-time quantification of trace gases in air and breath, known as selected ion flow tube mass spectrometry (SIFT-MS) (Smith & Španěl, 2005).

### 1.5.1 Brief working of SIFT-MS

It is based on the chemical ionisation of certain mass selected precursor ions with the sample gas molecules. The precursor ions[†] formed upstream - $H_3O^+$, $NO^+$, $O_2^+$ - are

[†] These specific ions are selected as they are capable of reacting with all organic compounds, many inorganic molecules and do not react with major air components.

introduced into an inert carrier gas, helium, at a known velocity. The sample gas is then introduced into this carrier gas/ion swarm at a known flow rate via a desired method. Reactions between the precursor ions and trace gases in the sample occur for a definite time period to generate characteristic product ions; these (including precursor ions) are then detected and counted downstream using a mass spectrometer, enabling quantification, as illustrated in Figure 1.4. Thus absolute concentrations of trace gases in a single exhaled breath can be quantified on-line and in real-time simultaneously at ppb levels. It can operate in two modes viz. full scan mode where it screens the entire spectrum and the multiple ion monitoring mode where specific product ions are targeted (Španěl & Smith, 1999; Smith & Španěl, 2005).

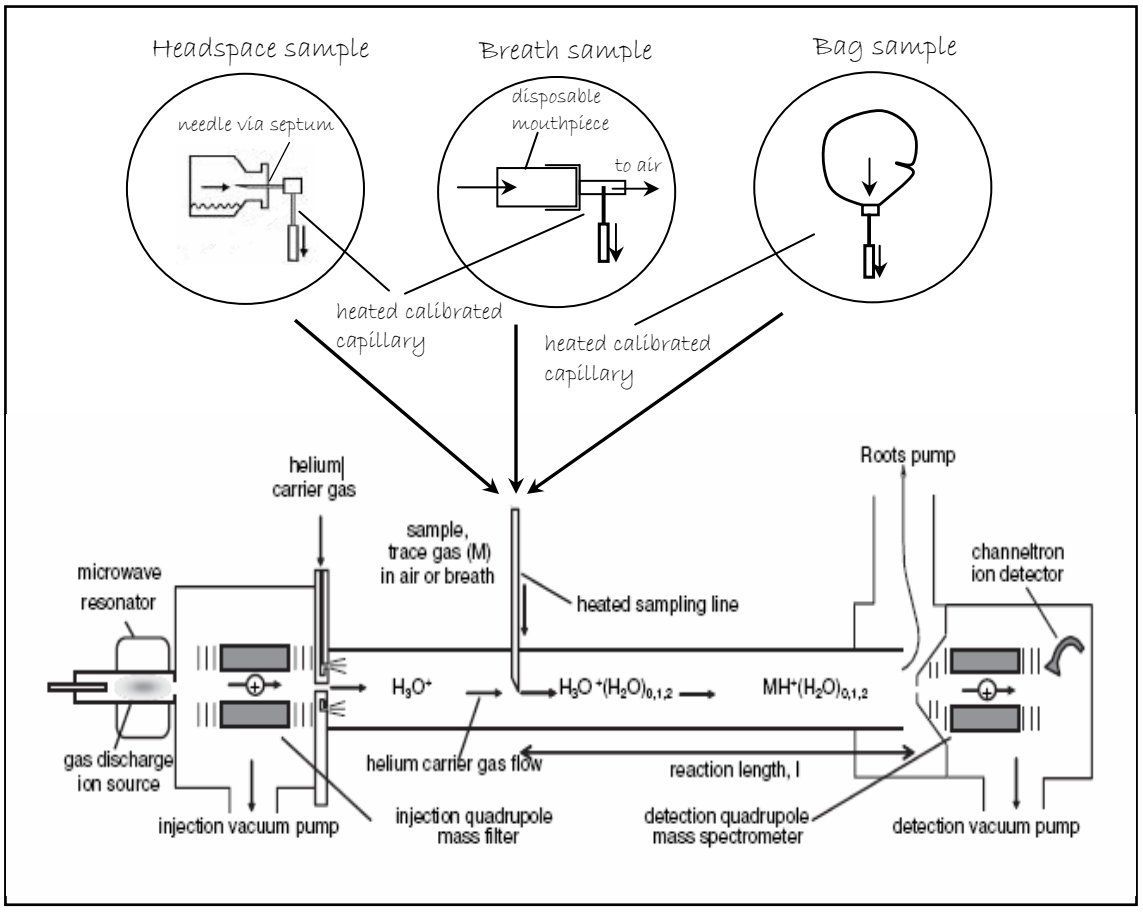



**Figure 1.4:** Schematic representation of the SIFT-MS instrument, showing an example reaction with $H_3O^+$ precursor ion, as adapted from Smith & Španěl (2005) and Turner *et al.* (2006).

### 1.5.2 Applications

These mass spectrometric techniques have been used in a variety of applications such as environmental, agricultural, medical, pharmaceutical and food sectors. GC-MS is also being extensively used in the fields of forensic science and law (Lee *et al.*, 2007).

Jelén & Grabarkiewicz-Szczesna (2005) found that the volatile profiles of *Aspergillus ochraceus* species using HS-SPME were similar regardless of their toxin producing ability. Although the conditions for maximal volatile production were different from those providing high amounts of ochratoxin, the volatile patterns could not differentiate toxigenic from non-toxigenic strains. However, in another study the different headspace profiles generated by *Penicillium roqueforti* fungal strains enabled differentiation between its toxin and non-toxin producing strains (Demyttenaere *et al.*, 2003)*;* whilst Nilsson *et al.* (1996) reported for the first time the presence of several mono and sesquiterpenes and alcohols in various *Penicillium* species.

Headspace analysis by using SPME with GC-MS has also been performed to determine microbial VOCs emitted from mould infested building materials. It was shown that similar compounds are produced although in different quantities enabling fungal species identification (Wady *et al.*, 2003). Kushalappa *et al.* (2002) managed to detect and discriminate six disease groups of potato tubers; while in another study it was possible to differentiate between pure and adulterated (with apple) strawberry samples based on the adulterant's aromatic compounds viz. hexanoic acid, 2-hexanal and α-farnesene (Reid *et al.*, 2004) using this technique.

Deng *et al.* (2004) determined volatile markers in human blood pertaining to lung cancer and showed that those markers (hexanal, heptanal) previously found in the breath of such patients actually originated in blood. Studies have also been carried out for analysis of metabolic disorders in children using urine samples, human skin emanations, human sweat, human breath as well as biological fluids for recognition of various diseases (Zlatkis *et al.*, 1981; Halket *et al.*, 1999; Di Francesco *et al.*, 2005; Zhang *et al.*, 2005; Dixon *et al.*, 2007).

Scotter *et al.* (2005) showed that VOCs from medically important fungi grown on media could be detected in real-time at levels of 100 ppb or lower using SIFT-MS and that volatile production was influenced by the growth medium. Studies have also been carried out on bacterial cultures *in vitro* using SIFT-MS. One study from cystic fibrosis patients, indicated that HCN especially from *Pseudomonas aeruginosa* could serve as a potential marker (Carroll *et al.*, 2005) whilst another identified compounds such as 3-methyl-butyl acetate, 4-methyl-1-pentadiene, 2-methyl-1-butanol and dimethyl polysulphides also to be associated with various bacterial species (Wang *et al.*, 2004). Antibiotic susceptibility in bacteria has also been demonstrated by inhibition of their volatiles (Allardyce *et al.*, 2006).

A number of studies have also been undertaken to determine potential cancer biomarkers with the help of SIFT-MS. Volatiles emitted from urine of patients suffering from bladder and prostate cancer showed elevated levels of formaldehyde as compared to normal patients (Španěl *et al.*, 1999). *In vitro* analyses of various tumour cell lysates detected the presence of formaldehyde (Kato *et al.*, 2000; Kato *et al.*, 2001). Smith *et al.* (2003) also detected the presence of acetaldehyde in lung cancer cell lines.

SIFT-MS has also been used for analysing breath in both healthy and unhealthy individuals (Diskin *et al.*, 2003; Turner *et al.*, 2006), monitoring food products and flavours (Španěl & Smith, 1999), sampling exhaust gases and *in situ* measurements of soil emissions (Smith & Španěl, 2005). However, these techniques are not portable and because of the economic cost and expertise needed are predominantly centralised research tools only. Thus it is essential to use such devices to identify target volatile biomarkers which can help interpret qualitative volatile fingerprints in simpler sensor-based diagnostic tools such as e-noses.

## *1.6 Volatile fingerprinting II: Olfactory technology and electronic noses*

For all living organisms, simple or complex, their response to chemicals in the surrounding environment forms an important basis for their daily survival. In humans, flavour is perceived by three distinct chemical senses: olfaction (sense of smell), gustation (sense of taste) and chemesthesis (response to irritants); with olfaction being the dominant of the three.

The presence of airborne VOCs is thus detected by humans via their olfactory and chemesthetic senses. It is essential to know what constitutes a smell or an odour, as the main human sensory system to detect flavour is olfaction. Odorant molecules are characterised by being polar, hydrophilic and light due to small molecular masses (up to 300 Da.). They can either be simple, comprising of a single molecule such as an alcohol or complex, consisting of a mixture of chemical constituents - for example perfumes or beverages. Nasal pungency, on the other hand is the unpleasant sensation like stinging, piquancy, irritation, burning or pricking that is felt by the chemoreceptor activation on the trigeminal nerve. Chemicals causing the latter are at far higher concentrations than

those eliciting odour sensations (Gardner & Bartlett, 1994; Craven *et al.*, 1996; Gardner & Bartlett, 1999; Cometto-Muñiz, 2002).

### 1.6.1 Analogy between human and machine olfaction

The human olfactory system is stimulated by odorant particles emitted from an object generating a sense of smell, which is drawn up into the nasal cavity, across the olfactory epithelium finally leading to the brain which processes the information and classifies the odour (Figure 1.5). Thus, it can be said to basically comprise of three vital elements: an array of olfactory receptor cells in the roof of the nasal cavity, the olfactory bulb located above the nasal cavity and the brain.

There has been strong dependence on human olfaction as a means of an analytical tool, although subjective, in industry for odour quality control and assurance of foods, flavours and fragrances. However, it tends to be costly as the human sensory panels are limited due to their inability to cope with assessing large number of samples per day and a constant need for highly trained personnel to distinguish the subtle nuances in complex mixtures. Besides, the human tendency to tire coupled with physical and mental states also put a restraint to such analysis. Moreover, there was need for continuous, regular monitoring and calibration of actual concentrations of substances for which gas chromatography-mass spectrometry were employed. These techniques are time consuming and at times inadequate, thus an enormous need exists for a system mimicking the human one (Gardner & Bartlett, 1994; Craven *et al.*, 1996; Pearce *et al.*, 2002).

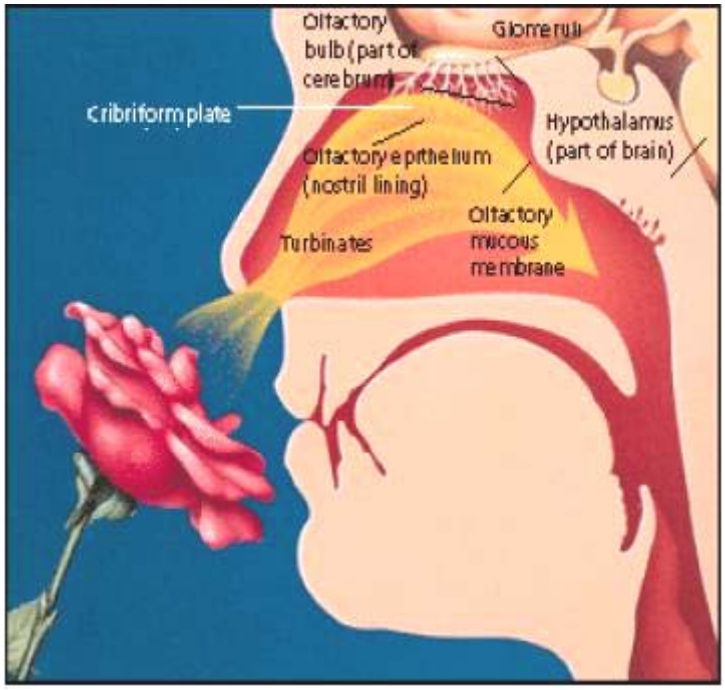



**Figure 1.5:** The process of human olfaction, adapted from Nagle *et al.* (1998).

The initial concept of a chemical sensor array in an artificial nose (electronic nose) for discrimination between odours was demonstrated by Persaud and Dodd (1982). Following which Gardner & Bartlett (1994) defined the electronic nose "as an instrument which comprises an array of electronic chemical sensors with partial specificity and an appropriate pattern-recognition system, capable of recognising simple or complex odours".

An electronic nose thus consists of three functional components mimicking the essential human elements, namely, a sensor array, a data pre-processor and pattern recognition (PARC) engine. The mechanism of the instrument is as follows: exposure of sensors to the volatiles from an odorant generating a transient response as the VOCs interact with the active material on the sensor surface, followed by signals being recorded and delivered to the processing unit during which time the sensors reach a steady state and are flushed with a washing gas. Prior to and subsequent to sample introduction, a

reference gas (air) is applied to the array preparing it for the measurement cycle. The response phase is the period when the odorant is applied while the recovery phase is when the system is flushed. Figure 1.6 shows the analogy between the human and artificial nose.

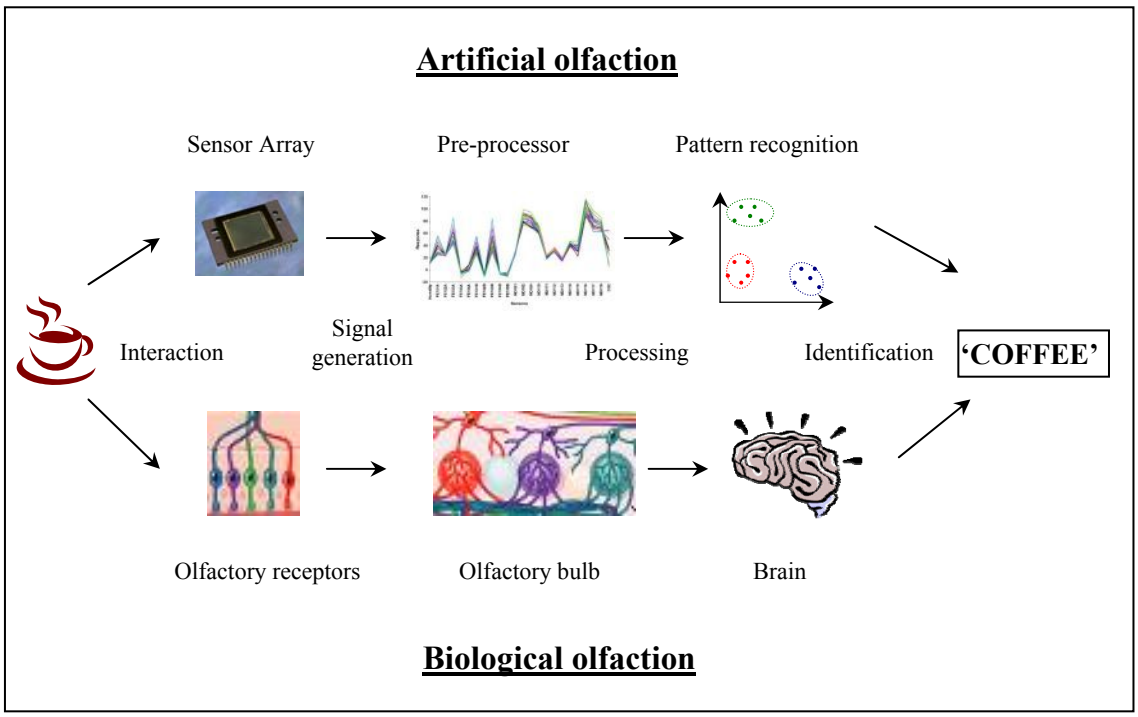



**Figure 1.6:** Analogy between artificial (electronic) and biological olfaction, adapted from Hines *et al.* (2002).

The processing and identification stages can be considered to comprise of sub-stages until a final decision is reached. Pre-processing involves normalising sensor responses, manipulating baselines to ensure reduction in sample variations, minimising sensor drift and compressing transient array responses. The next stage is feature extraction where the dimensionality of the measurement space is reduced and relevant information extracted for pattern recognition which employs multivariate analysis. Similarly, in the human system noise reduction by means of signal compression and output amplification

enhances the sensitivity and selectivity of olfaction. Finally, a classifier determining the category to which the sample belongs is implemented. Classifications can be performed using techniques such as Bayesian classifiers, K nearest neighbours (KNNs) or artificial neural networks (ANNs) (Nagle *et al.*, 1998).

### 1.6.2 Sensor technologies

A fundamental aspect of a sensor array is sensitivity to various chemical compounds, i.e. it is non-specific, along with high stability, high reproducibility, short reaction and recovery time. It is essential that the sensors on the array have varying sensitivities to the same substance so that different odours have a distinct response pattern across the sensors enabling an unknown odour to be identified. Each sensor thus has a unique response profile to the spectrum of odorous molecules resulting in alteration of its physical properties such as mass, electrical conductivity or capacitance that can be measured directly or indirectly (Nagle *et al.*, 1998; Nanto & Stetter, 2002). Sensors are classified based on their operating principle, each of which has a different sensitivity and selectivity. The various sensor technologies are described below.

a) Conducting Polymer (CP): They exhibit change in conductivity (resistance) on exposure to volatile gases that bond to the polymer backbone (polypyrrole, polythiophene or polyaniline). They possess reversible physicochemical properties, are extremely sensitive (0.1–100 ppm), respond to a broad range of organic vapours and operate at ambient temperatures. However, they are susceptible to humidity and poisoning, can drift over time and show undesirable batch variations due to difficulty in their fabrication.

b) Metal Oxide Semiconductor (MOS): These rely on change in resistance when exposed to gaseous molecules and are made of oxides of tin, zinc, tungsten operating at high temperatures, 200°C-500°C. Normally, oxygen is adsorbed to the surface which forms a potential barrier restricting electron flow; on exposure to volatile gases oxidation occurs, allowing electron flow thus decreasing resistance. Their sensitivity and selectivity can be modified by doping with catalysts such as platinum or palladium or changing the operating temperature. They are very sensitive (5-500 ppm), resistant to humidity but are prone to drift over time and to poisoning by sulphur containing compounds and weak acids due to irreversible binding.

c) Metal Oxide Semiconductor Field Effect Transistor (MOSFET): When VOCs come in contact with a catalytic metal it produces a reaction, the products of which diffuse through the gate changing its surface potential. The voltage shift depends on the gas concentration. The sensitivity and selectivity can be enhanced by varying the thickness/type of metal catalyst and changing the operating temperature, usually 100°C-200°C. They too are susceptible to drift similar to conductivity sensors.

d) Quartz Crystal Microbalance (QCM): Also known as a bulk acoustic wave device is made of a polymer-coated resonating quartz disc, vibrating at a characteristic frequency (10-30 MHz). Adsorption of volatile molecules to the polymer surface increases mass of the disc, thus reducing the resonance frequency. The decrease is inversely proportional to mass of odorant adsorbed. The sensor selectivity is dictated by the thickness of the coatings, while sensor sensitivity can be affected by changes in temperature, humidity and flow conditions these can however be made negligible.

e) Surface Acoustic Wave (SAW): These too measure changes in mass but require waves to travel over the surface of the device. They operate at higher frequencies (100-1000MHz) and thus generate a larger change in frequency. They do suffer from reduced long term stability and are highly sensitive to humidity.

f) Optical fibres: These employ glass fibres coated with thin chemically active materials on their sides or ends that contain immobilised fluorescent dyes in an organic polymer matrix. Interaction of volatiles with a light source alters the dye polarity causing a shift in the emission spectrum (colour change). Wide sensitivities are obtainable owing to availability of different dyes however they have a limited lifetime because of photobleaching (Nagle *et al.*, 1998; Gardner & Bartlett, 1999; Jurs *et al.*, 2000; Nanto & Stetter, 2002).

Apart from those mentioned above, many more technologies are emerging making use of ligand binding properties, aromatic compounds and improving optical sensors. Another optical phenomenon made use of is surface plasmon resonance (SPR) where a change in the refractive index of a sample's surface is measured (Nanto & Stetter, 2002). Discotic liquid crystals (DLC) consisting of an aromatic core surrounded by hydrocarbon side chains are very sensitive to the presence of volatile molecules, especially non-polar species, and insensitive to humidity (Turner & Magan, 2004). Recently, colorimetric arrays have been developed making use of metalloporphyrins that induce colour changes on the binding of metal ions present in volatile compounds. Furthermore, these compounds are resistant to humidity and have high sensitivity (Suslick & Rakow, 2001). Table 1.3 highlights the main attributes of these sensors.

**Table 1.3:** Summary of the sensor technologies for electronic nose, adapted from Nagle *et al.* (1998) and Nanto & Stetter (2002).

| Sensor Type | Operation Principle | Fabrication Method | Sensitivity | Advantages | Disadvantages |
|---|---|---|---|---|---|
| Metal oxide (MOS) | Conductivity (Conductance) | Microfabricated | 5-500 ppm | Inexpensive, microfab. | Operates at high temp. |
| Conducting polymer (CP) | Conductivity (Conductance) | Microfabricated, electroplating, screen printing | 0.1-100 ppm | Operates at room temp., microfab. | Very sensitive to humidity |
| MOSFET | Capacitive charge coupling | Microfabricated | ppm | Integrated with electronic interface circuits | Odorant reaction product must penetrate gate |
| Quartz crystal microbalance (QCM/QMB) | Piezoelectricity | Screen printing, wire bonding, MEMS‡ | 1 ng mass change | Well understood technology | MEMS fab., interface electronics |
| Surface acoustic wave (SAW) | Piezoelectricity | Screen printing, microfab. | 1 pg mass change | Differential devices quite sensitive | Interface electronics |
| Fluorescence, chemolumin-escence | Optical – intensity/spectrum (fibre optic) | Dip coating, MEMS, precision machining | Low ppb | High electrical noise immunity | Restricted availability of light sources |
| SPR§ | Optical – refractive index | Screen printing, microfab., dip coating | Low ppb | High electrical noise immunity | Expensive |
| Gas chromatograp-hy | Molecular spectrum | MEMS, precision machining | Low ppb | Potential analyte accuracy | Sample concentration required |
| Mass spectrometry | Atomic mass spectrum | MEMS, precision machining | Low ppb | Potential analyte accuracy | Sample concentration required |
| Light spectrum | Transmitted light spectrum | MEMS, precision machining | Low ppb | Sample not consumed | Requires tuneable quantum well devices |

‡ MEMS → microelectromechanical system
§ SPR → Surface Plasmon Resonance

### 1.6.3 Odour sampling mechanisms

Turner and Magan (2004) outlined the crucial elements for the efficacy of an electronic nose. The first being consistency and reproducibility in sampling, which would involve standardising parameters such as humidity, temperature and sample size to ensure reliability of data sets which can then be analysed with statistical confidence. Secondly, the ability of pattern recognition techniques to analyse large data sets effectively. Individual sensors generate significant amounts of data by extracting information from the various parameters in a typical sensor response, which can be effectively managed by using complex multivariate analysis. This is discussed in the following section whilst the sampling methods are briefly discussed below.

**Sampling methods:** Numerous methods can be employed for the sampling process comprising sample uptake, its conditioning and transfer to the analytical equipment. However, care needs to be taken to avoid altering the headspace composition by utmost efficiency whilst sampling (Web-resource, 2007). The two main odour sampling methods used in electronic nose systems are:

a) Static Headspace Analysis (SHA): The desired sample is placed in a vial which is set aside for a specific time period to allow the headspace to be saturated with the odours, i.e. until it reaches equilibrium. The headspace is then transferred directly to the sensor array chamber using an injector either manually or by means of an autosampler. This form of delivery helps in reducing the variations in sample temperature, injection rate and concentration of the headspace.
b) Flow Injection Analysis (FIA): It is typically a computer automated method where a background gas (usually clean air) is constantly being pumped into the sensor chamber. Before the odorous gas (i.e. the sample headspace) reaches the sensors, it

is injected into the background gas. The ratio of the mixture of background gas to odour gas can be precisely controlled (Craven *et al.*, 1996; Gardner & Bartlett, 1999).

SHA is however the more popular and low cost method. There are other variations of such a closed system such as diffusion, permeation and bubbling gas (e.g. air) to generate vapour (Nakamota, 2002). However, for measuring a sample with very low concentrations of volatile compounds preconcentration of the sample prior to its investigation might be necessary. This can help improve sensitivity of the sensing process, take advantage of large sample volumes and reach lower threshold limits. Various methods for preconcentration are described in the literature such as thermodesorption, solvent extraction and solid phase extraction (Web-resource, 2007).

### 1.6.4 Pattern recognition and conventional multivariate statistics

The crucial elements in implementing, developing and commercialising electronic noses are pattern recognition (PARC) algorithms and efficient data processing techniques (Hines *et al.*, 2002). Prior to proceeding to such analyses, pre-processing signals from raw data can be beneficial in reducing factors such as sensor drift and noise and removing redundant features such as unsuitable sensors. It can be done by scaling or normalising sensor responses in order to eliminate or reduce the effect of concentration related fluctuations (Craven *et al.*, 1996; Dickinson *et al.*, 1998; Otto, 1999).

Subsequent multivariate analyses that are employed by pattern recognition algorithms aim at the grouping and classification of objects or samples as well as modelling relationships between different data (Otto, 1999). These can be divided into two approaches; one based on obtaining an overview of the data, i.e. conventional statistics

and the other based on human cognition - thus biologically motivated. These can be further subdivided into supervised and unsupervised methods (Gardner & Bartlett, 1999). Unsupervised PARC techniques are best for qualitative applications such as exploring relationships in data by searching for similarities. They make no prior assumptions about the data i.e. they are non-parametric. These include principal component analysis (PCA), cluster analysis (CA) and Kohonen networks or self-organising maps (SOM) (Pearce, 1997; Otto, 1999; Brereton, 2003).

Supervised PARC methods on the other hand, are mostly aimed at classification. It requires *a priori* knowledge of known groups in the form of a training set and then attempts to classify an unknown sample within the known groups. It is, of course, always essential to initially establish whether the measurements are acceptable to fit the predetermined groups (Pearce, 1997; Brereton, 2003). These include discriminant function analysis (DFA), K-nearest neighbours (KNN), soft independent modelling of class analogies (SIMCA) and artificial neural networks (ANNs) (Pearce, 1997; Otto, 1999).

a) Principal component analysis (PCA): It is an effective unsupervised pattern recognition technique which helps identify general relationships within the data. This is done by determining the variation in a dataset (seeks a direction in space which captures maximal variance) in the form of a set of new uncorrelated variables i.e. principal components, which are linear combinations of the original variables. These principal components (PCs) are obtained in decreasing order of importance, where the first PC has the maximum variance followed by the second PC having the maximum of the remaining variance and so on. Thus, it reduces the dimensionality

of the data by preserving maximum information with minimum number of variables (Gardner & Bartlett, 1999; Everitt & Dunn, 2001; Brereton, 2003). Mathematically it can be represented as:

$$X = T \cdot P + E \qquad \text{... (Eqn 1.1)}$$

where X is the original data matrix, T is the scores matrix having same number of rows as X, P is the loadings matrix with the same number of columns as X and E is the error matrix.

b) Cluster Analysis (CA): This is another unsupervised pattern recognition technique that enables one to establish or determine the relationships between samples and sample groups by finding the natural groupings within the dataset. One form is agglomerative hierarchical clustering, which is a two step process. First the similarities between the samples are determined by measuring the distances between them, using various distance measures such as the Euclidean distance given below:

$$d_{ij} = \sqrt{\sum_{k=1}^{N} (x_{ik} - x_{jk})^2} \qquad \text{... (Eqn 1.2)}$$

Secondly, the samples are then individually linked together to form clusters using a variety of linkage algorithms, for example Ward's or complete linkage. The result of which is represented by means of a dendrogram i.e. tree-diagram (Gardner & Bartlett, 1999; Brereton, 2003).

c) Discriminant Function Analysis (DFA): It is a parametric and supervised pattern recognition method that addresses the issue of discriminating classes of samples and then allocating new samples to respective classes based on the previously trained information.

d) Partial Least Squares (PLS): It is a supervised regression technique that incorporates some PCA properties and is used for prediction purposes. Unlike PCA, it models both the independent (sensor responses) and dependent (e.g. concentrations) variables where it determines the correlation between the two variables (Gardner & Bartlett, 1999).

### 1.6.5 Artificial Intelligence

Artificial intelligence (AI) is an area of computer science committed to the production of sophisticated software capable of intelligent computations similar to those that the human brain routinely performs. One component, being dedicated to computer programs that simulate the manner in which the human brain processes information primarily aimed at problem solving, called artificial neural networks (ANN; Agatonovic-Kustrin & Beresford, 2000). Thus, a neural network can be defined as a model of reasoning based on the human brain due to which it is used widely for pattern recognition. It is known to possess remarkable information processing characteristics for instance nonlinearity, high parallelism, robustness, fault and failure tolerance, learning, ability to handle imprecise and fuzzy information and a capability to generalise (Basheer & Hajmeer, 2000). Therefore, they have the capability to solve complex real life problems for example pattern classification, clustering, function approximation, optimisation, forecasting and prediction, association, nonlinear system modelling, memory and control (Basheer & Hajmeer, 2000; Sun *et al.*, 2003).

Just as the human brain – an excellent pattern recognition tool - consists of a densely (≈ 10 billion) interconnected set of nerve cells or neurons; an ANN too comprises of a number of very simple processors, also called neurons, analogous to those in the brain

(Negnevitsky, 2002). Figure 1.7 shows the analogy between the biological and artificial networks.

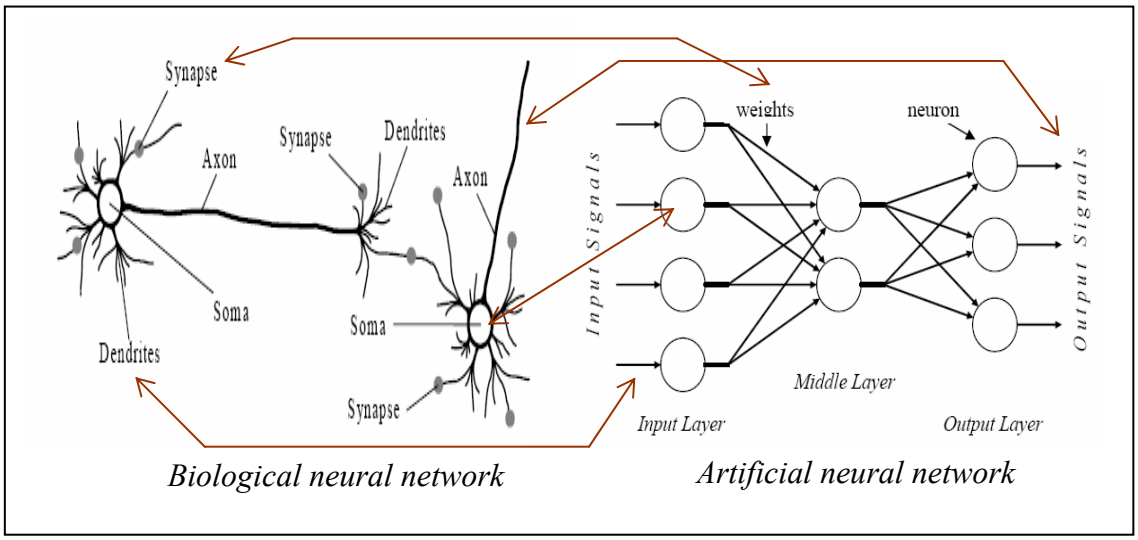



**Figure 1.7:** Analogy between artificial and biological neural networks, adapted from Negnevitsky (2002).

Most ANNs have a multi-layered architecture where the first layer - *Input layer* - has no computing ability and is used for input of independent variables. Each neuron/node in this layer comprises of a single inserted variable. The last layer - *Output layer* - processes the outcome of the dependent variables and can have one or many neurons depending on the desired output. The second layer - *Hidden (Middle) layer* - provides interconnections between the input and output layers. There can be more than one hidden layer each with many neurons, but the number depends on the complexity of the problem. The neurons process information based on the weighted inputs using their transfer (activation) functions and sends out outputs. The weights are critical to the network and are involved in the learning process. The net input to the next layer's neuron is the sum of the weighted outputs of the previous layer (or the sum of the initial weighted inputs for the first hidden layer). It is given by the following equation:

$$X = \sum_{i=1}^{n} w_i x_i + b \qquad \ldots \textit{(Eqn 1.3)}$$

where $X$ is the net weighted input, $w_i$ is the weight of input i, $x_i$ is the value of input i, $n$ is the number of neurons and $b$ is the bias (threshold value). The net input is then passed to a transfer function which determines the output value. The most commonly used transfer function is the sigmoid function (especially in back propagation networks). The actual output $Y$ (using the sigmoid function) is represented as:

$$Y_i = \frac{1}{1 + e^{-x}} \qquad \ldots \textit{(Eqn 1.4)}$$

The transfer function helps introduce non-linearity to the network. Furthermore, artificial neurons receive excitatory and inhibitory inputs just like real neurons. Excitatory inputs cause the summing mechanism of the next neuron to add while the inhibitory inputs cause it to subtract. A neuron can also inhibit other neurons in the same layer, termed as 'lateral inhibition' (Agatonovic-Kustrin & Beresford, 2000). ANNs are thus characterised by their neural connections (network architecture), the transfer function used by the neurons and the learning algorithm that specifies the procedure for adjusting weights (Zupančič Božič *et al.*, 1997; Negnevitsky, 2002).

Learning is the most fundamental aspect involving adaptive mechanisms allowing ANNs to learn from experience, by example or analogy; which can be improved over time (Negnevitsky, 2002). ANNs can learn either by supervised methods (e.g. backpropagation) or unsupervised methods (e.g. self organising maps).

### 1.6.6 Applications

Electronic noses have targeted applications, present and potential, ranging from food and drugs to medical diagnostics, environmental monitoring, safety and security and for military use (Göpel, 2000).

**a. Food industry:** In the food industry, the technology has been mainly used for testing freshness of foodstuff, quality control, process operations, minimising batch variations, screening the raw materials used or contamination.

Many of the early investigations were done extensively on cereal grains, especially for the early detection of spoilage fungi and quality changes by means of odorous off-taints (Magan & Evans, 2000). Keshri *et al.* (1998) could discriminate between *Penicillium* and *Eurotium* species in agro-based substrates prior to visible growth based on their *in vitro* volatile production patterns. Successful attempts have been carried out to differentiate between toxic and non-toxic strains of *Fusarium* in cereal grains (Keshri & Magan, 2000; Falasconi *et al.*, 2005a). The latter could classify the strains based on their toxin producing capability.

Microbial spoilage caused by bacteria, yeast and filamentous fungi as well as enzymatic spoilage of bakery products could be detected earlier based on their volatile profiles and were found to be better than traditional enzymatic assays (Keshri & Magan, 2000; Needham *et al.*, 2005). Similarly, studies have been carried out in dairy products such as determining the shelf life of milk (Labreche *et al.*, 2005), detecting spoilage micro-organisms (bacteria and yeast species) in milk (Magan *et al.*, 2001) and monitoring the ripening and quality control of Danish blue cheese (Trihaas & Nielsen, 2005) using

sensor arrays in conjunction with artificial neural networks. Marsili (1999) studied off-flavours in milk caused by bacteria as well as physical methods such as spiking with copper and irradiation, by developing a new electronic nose system comprising solid phase micro-extraction, mass spectrometry and multivariate analysis.

However, this technology has also been applied on other foodstuff such as fruits, meat, fish, wines – both red and white, coffee, extra virgin olive oils and white truffles (Deisingh *et al.*, 2004; Falasconi *et al.*, 2005b; Falasconi *et al.*, 2005c; Lozano *et al.*, 2005; Cimato *et al.*, 2006; Garcia *et al.*, 2006; Rajamäki *et al.*, 2006).

**b. Environmental monitoring:** Over the years, pollution has increased at an alarming rate, thus resulting in an urgent need to constantly monitor the amount of pollution in order to prevent detrimental effects. Applications are therefore designed that mainly involve processes for quality control, monitoring pollution of air and water sources, detection of excess volatiles in housing or work complexes and monitoring effluent release from plants.

Studies for monitoring indoor air quality control (especially for $NO_2$ and CO gases) as well as detection of fungal contamination on various building materials have been successfully carried out using metal oxide sensors and fuzzy logic algorithms (Zampolli *et al.*, 2004; Kuske *et al.*, 2005).

Non-specific sensor arrays have been used for on-line monitoring of industrial discharges in domestic wastewater samples (Bourgeois & Stuetz, 2002) as well as detecting a range of odour concentrations from sewage works (Stuetz *et al.*, 1999). Nake

*et al.* (2005) investigated two different electronic nose systems for the *in situ* monitoring of outdoor air from wastewater treatment plants while Baby *et al.* (2000) examined the contamination of water sources by insecticides and products from the leather industry based on their odours. Detection of microbial and chemical contamination of potable water has also been studied (Canhoto & Magan, 2003; Canhoto & Magan, 2005).

Sensor arrays have also been used in the automotive industry for quality control monitoring (such as materials for seat manufacture and air quality) (Morvan *et al.*, 2000); in libraries for detecting fungal contamination in papers (Canhoto *et al.*, 2004); identifying the different blends of gasoline (Brudzewski *et al.*, 2006) and identifying wood samples and relationships between plant species (Wilson *et al.*, 2005).

**c. Medical Applications:** Although odours have been used for centuries for diagnosing diseases, it has been restricted to certain characteristic scents recognised by humans. Advances in artificial olfaction have only recently led to the identification and early detection of bacterial infections as well as non-infectious diseases by monitoring odours from body fluids and breath (Saini *et al.*, 2001).

Lately, there has been an increasing need for early identification and detection of microbial infections in hospitals. Ventilator associated pneumonia, a lethal infection that occurs in intensive care units is caused by a variety of micro-organisms, especially bacteria. A recent study has identified these organisms on the basis of their gaseous products using electronic nose and reduced the analysis time by 50 percent (Serneels *et al.*, 2004). In another case, Dutta *et al.* (2005) identified *Staphylococcus* species such as

methicillin-resistant *S. aureus* (MRSA), methicillin-susceptible *S. aureus* (MSSA) and coagulase negative staphylococci (C-NS) from swab samples of infected patients using a sensor array with a radial basis function network with 99.69% accuracy.

Successful attempts have also been made in early diagnosis of tuberculosis in animals such as badgers and cattle using serum (Fend *et al.*, 2005). Similarly, Pavlou *et al.* (2004) detected *Mycobacterium tuberculosis* in cultured human sputum samples directly or subsequent to enzymatic treatment for enhancing bacterial growth. Sensor technology has also been applied for identifying bacteria causing urinary tract infections (Pavlou *et al.*, 2002a) and gastrointestinal diseases (Pavlou *et al.*, 2000); detecting bacterial contamination in biomedical samples, for example, blood and urine (Yates *et al.*, 2005) and as a screening test for bacterial vaginosis (Chandiok *et al.*, 1997).

Gas sensors have proved to be a useful non-invasive technique for detecting non-infectious diseases. Di Natale *et al.* (2003) made use of non-selective sensors coated with metalloporphyrins for immediate breath analysis to identify lung cancer. In a similar manner, lung cancer could be diagnosed on the basis of the eleven validated volatile markers from their simultaneous pathological study (mostly aromatic and alkane derivatives), qualitatively and quantitatively (Chen *et al.*, 2005). *In vitro* distinction between different tumour cell lines as well as normal fibroblast and smooth muscle cell lines has also been shown (Gendron *et al.*, 2007).

A study has been carried out to distinguish blood and urine odour types dependent on the major histocompatibility complex (MHC) expression in different mouse strains including detection of individual odour types of human sera (Montag *et al.*, 2001).

Recently, Balseiro & Correja (2006) proposed that VOCs produced by tumour cells are products of MHC genes based on supporting evidences. Thus, suggesting that soluble human leukocyte antigen (HLA) molecules in body fluids (blood, urine and sweat) can produce volatiles that can function as a diagnostic marker for cancer using electronic noses.

Moreover, sensor array systems serve to be a suitable tool for online monitoring of renal dialysis, since pre and post dialysis blood could be easily discriminated (Fend *et al.*, 2004); differentiating between cerebrospinal fluid (CSF) and serum, useful in certain clinical circumstances (Aronzon *et al.*, 2005) and testing acetone concentration in expired breath to detect diabetes (Wang *et al.*, 1997). In the latter, it was suggested that analysis of breath after a meal might be more significant as in diabetics the concentration of acetone would remain elevated whilst in normal individuals it would return to normal levels.

Electronic noses have also found their way in the pharmaceutical industry where they can be used for screening raw materials, testing people in critical occupations for drug use or abuse and testing breath and urine for targeted illegal compounds or by-products (Nagle *et al.*, 1998). Zhu *et al.* (2004) have demonstrated that the instrument can be used to identify flavours in pharmaceutical products; to test the identity of flavoured raw materials and flavoured solution formulations (i.e. discerning fresh and old samples as well as those from different batches) and for qualitative analysis of flavours in oral solution formulations.

## *1.7 Aims and Objectives*

The main aim of this study is to implement volatile fingerprinting, primarily using e-nose technology in specific medical settings and determine its potential for early identification and diagnoses of diseases or infections.

The objectives set out to carry these aims were as follows:

A. Dermatophytes:

- Evaluate the potential of two electronic nose systems based on conducting polymer and metal oxide sensor arrays for detecting volatile production patterns produced by the dermatophytes - *Trichophyton* species.
- Determine the optimum time at which effective discrimination between four different *Trichophyton* species could be achieved using the best sensor array system.
- Determining the sensitivity threshold for detection of two individual species, *T. mentagrophytes* and *T. rubrum.*
- Discriminate between species causing human (*T. mentagrophytes* and *T. rubrum*) and animal infections (*M. canis*).
- Identify any similarity or differences between strains of the two most common species - *T. mentagrophytes* and *T. rubrum.*
- Develop a computational model to classify samples based on the respective fungal species.
- Identify compounds responsible for the discrimination of the fungal species using mass spectrometric methods (i.e. biomarker detection).
- Temporal effects of the antifungal against the two common dermatophytes, *T. mentagrophytes* and *T. rubrum.*

- Test the potential of the e-nose for antifungal screening against the two dermatophytes.

B. Ventilator associated pneumonia:

- Evaluate the potential of electronic nose to differentiate between samples obtained from healthy and unhealthy patient groups.
- Determine the different organisms causing the disease in clinical samples based on the volatile profiles.
- Correlate the hospital's microbiology results to the e-nose profiles.
- Differentiate between the different bacteria causing the disease *in vitro* based on lab cultures.

C. Oesophageal cancer:

- Determine the diagnostic potential of e-nose with clinical sample groups and correlate these with the routine endoscopy findings from the hospital.
- Discriminate between cancerous and normal oesophageal cell lines *in vitro*.
- Identify possible biomarkers from the oesophageal cell lines using mass spectrometric methods.

The overview of the thesis is schematically represented in Figure 1.8. Chapters 2 to 5 deal with the three medical aspects described above: fungal, bacterial and oesophageal cancer respectively; each of which are presented independently. An integrated discussion is presented in Chapter 6 which attempts to draw out some common features from all the phases of this work which is followed by a final set of conclusions.

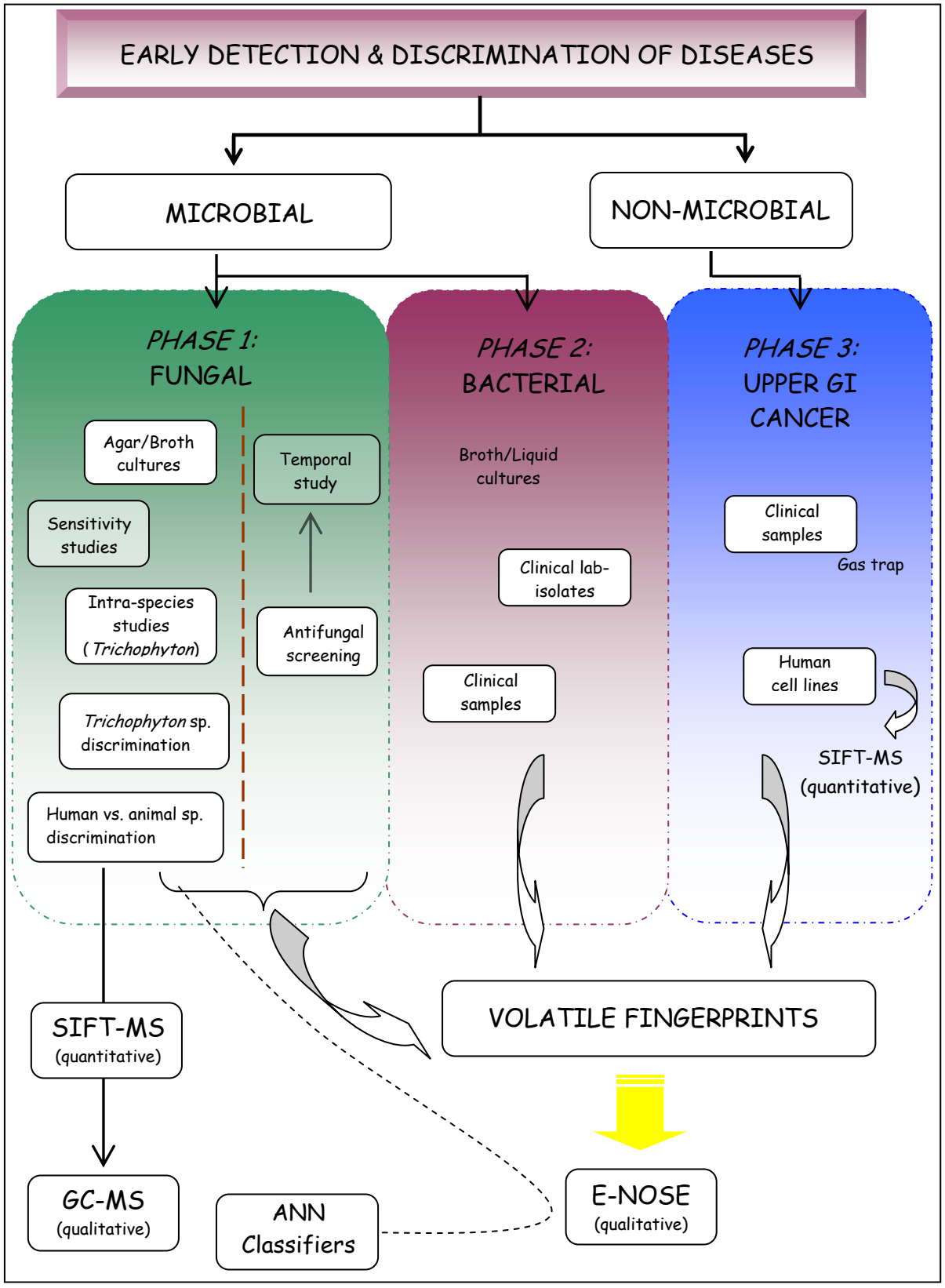



**Figure 1.8:** Schematic representation of the phases presented in the thesis.

# *Chapter 2*

# *EARLY DETECTION OF MEDICALLY IMPORTANT FUNGI*

## *2.1 Introduction*

Fungal volatiles have been substantially researched over the past few years. Their main focus however lay within the food and feed industry, because massive economic losses could be brought about due to fungal spoilage resulting in deteriorating food quality. Studies then demonstrated the presence of key organic compounds such as 1-octen-3-ol, 3-methyl-1-butanol and sesquiterpenes which were detected primarily by gas chromatography and mass spectrometry (Magan & Evans, 2000).

Subsequently, researchers began using simpler detection methods of microbial species such as electronic noses (Keshri *et al.*, 2002; Needham *et al.*, 2005), which were then combined with artificial neural networks for classification and prediction. Evans *et al.* (2000) used an electronic nose with a radial basis function network for predicting wheat quality using artificially contaminated and commercial samples and achieved a high rate of success. In another study, tea quality was predicted with almost precise accuracy using e-nose sensor data and radial basis function or probabilistic neural networks (Dutta *et al.*, 2003).

These e-nose techniques have only recently been used in medical diagnosis but mainly for certain bacterial infections (Pavlou *et al.*, 2002a; Pavlou *et al.*, 2004) or breath analyses for lung cancer (Di Natale *et al.*, 2003; Chen *et al.*, 2005). The inclusion of probabilistic neural networks has led to good prediction capabilities of e-noses with high accuracy (Dutta *et al.*, 2002; Dutta *et al.*, 2005). However, not much attention has been given to filamentous fungal infections, especially those pertaining to medically relevant filamentous species.

Dermatophytosis is a non-life threatening infection that occurs in the keratinised tissues of humans and animals caused by a group of filamentous fungi. The traditional so-called gold standard identification procedures involve cultures, biochemical tests and microscopy. These are however, time-consuming, tedious and require skilled microbiologists. Advances in molecular diagnostic methods have resulted in improved detection of these fungi (Binstock, 2007), but are not feasible for routine use in clinics and are uneconomical due to the costs of certain associated equipment and materials. No studies to date have been undertaken using volatile profiles of these fungi with electronic noses to try and discriminate between species. Clinically, early detection of the species would result in appropriate antifungal administration.

This study assessed the potential of volatile profile patterns of the dermatophytes generated by electronic noses in differentiating these fungal species and determining any similarities or differences amongst strains of the two main species. It also attempted to identify the volatiles present in these pathogenic fungi.

## *2.2 Materials and Methods*

### 2.2.1 Fungal species, strains & growth media

Studies were carried out using type cultures from two of the dermatophyte genera viz. *Trichophyton* and *Microsporum*. Four important anthropophilic *Trichophyton* species namely *T. mentagrophytes* (NCPF-224)*, T. rubrum* (NCPF-115 and strain D12), *T. verrucosum* (NCPF-685) *and T. violaceum* (NCPF-677) and an important zoophilic species *M. canis* (MC-177) were selected. In addition, four strains of *T. mentagrophytes* (M-61, M-62, M-63 and M-64) and three strains of *T. rubrum* (R-55, R-57 and R-59) were also used. Further details of these fungal strains can be found in Appendix A, A.1.

For *in vitro* studies the cultures were grown on two types of agar-based media and in a liquid broth.

**a) Agar based media**

- Sabouraud Brain Heart Infusion (SABHI) Agar was prepared by mixing Brain Heart Infusion (BHI) agar (Oxoid) and supplementing it with the required amount of Glucose (Acros Chemicals) with the addition of a small amount of the antibiotic, Chloramphenicol (Sigma) [47 g $l^{-1}$ BHI + 38 g $l^{-1}$ Glucose] (Kern & Blevins, 1997).
- Sabouraud Dextrose Agar (SDA) was prepared in house by mixing 10 g $l^{-1}$ Mycological peptone (Amersham), 40 g $l^{-1}$ Glucose (Acros Chemicals) and 15 g $l^{-1}$ Agar technical no. 3 (Oxoid). A small amount of the antibiotic, Chloramphenicol (Sigma) was also added.

**b) Liquid broth**

Sabouraud Dextrose (SD) broth was also prepared in house in the similar manner as SDA, described above, without the addition of agar.

### 2.2.2 Fungal growth curves

Individual spore suspensions of all the fungal species and strains were prepared and these were then used to centrally point inoculate agar (SDA) plates. Three replicates per fungal species and strains were incubated at 25°C. Growth rate measurements were determined by measuring the diameter of the colony in two directions, perpendicular to each other for about four weeks.

### 2.2.3 Species and strain discrimination

Three to four week old actively growing cultures of each of the four *Trichophyton* species and the *Microsporum* species were harvested using a sterile loop to prepare spore suspensions in sterile 10 ml Tween 80 (Acros Chemicals) and RO water. 20 to 25 replicate agar plates were inoculated with 250 µl of the inoculum – in the range of $10^{5-6}$ spores $ml^{-1}$ for the *Trichophyton* experiments and $10^{6-7}$ spores $ml^{-1}$ for the second set involving the animal pathogen, measured using a haemocytometer, and spread plated on the agar surface as a spore lawn. The plates were incubated at 25°C in the dark for 24-120 hours and every 24 hours five replicates of each species were destructively sampled. Blank agar plates were used as controls.

Four 2 cm diameter agar discs were placed in 25 ml vials and/or Universal bottles and left to equilibrate for one hour at 25°C; thereafter the headspace generated was analysed using two sensor array systems. Studies were carried out using both agar media and were repeated at least twice.

For initial liquid culture studies only the two faster growing *Trichophyton* species of the four i.e. *T. mentagrophytes* and *T. rubrum,* were used (two-three week old cultures). 50 ml of Sabouraud Dextrose broth was placed in 250 ml Erlenmeyer conical flasks (five replicates of each) and inoculated with a fungal spore suspension (approximately $10^6$ spores $ml^{-1}$). They were then plugged with cotton wool, loosely covered with aluminium foil and left to incubate at 25°C on a rotary shaker at 150 rpm for 24-96 hours. Every 24 hours 5 ml of a representative sample was transferred into vials and left to equilibrate for an hour before analysing the headspace. Sterile liquid medium was used as a control.

Similar agar based experiments were carried out using numerous strains of *T. mentagrophytes* and *T. rubrum* which were grown for about three weeks prior to making spore suspensions. Replicate SDA agar plates (25-30) were inoculated (roughly $10^{6-7}$ spores $ml^{-1}$), incubated, sampled and analysed as stated previously. Controls were in the form of blank agar plates. Headspace measurement was however carried out using only one of the e-nose systems (AS Nordic).

### 2.2.4 Sensitivity thresholds for detection

Spore suspensions from two-three week old growing cultures of *T. mentagrophytes* and *T. rubrum* were made and the initial concentrations were determined using a haemocytometer. Serial dilutions, generally 10 or 100-fold, were then prepared from the stock solutions in order to obtain treatments of $10^1$, $10^3$, $10^5$ and $10^7$ CFUs (spores) $ml^{-1}$.

For agar based studies, 250 µl from each treatment were spread plated on at least 25 replicate agar plates. These were inoculated and analysed as described previously.

For liquid broth cultures, initially 130 ml of the Sabouraud broth medium was placed in 250 ml Erlenmeyer conical flasks and inoculated with the stock solution to obtain a concentration of $10^7$ spores $ml^{-1}$. Subsequently, 100-fold serial dilutions were carried out to obtain treatments of $10^5$, $10^3$, and $10^1$ spores $ml^{-1}$. These were inoculated and analysed as before. Alternatively, 5 ml of suspension from each of the treatment flasks was pipetted into Universal bottles and incubated at 25°C on a shaker at 150 rpm. The replicates/treatments were sampled as described previously.

### 2.2.5 Mass spectrometric systems

Two different mass spectrometric techniques, gas chromatography-mass spectrometry (GC-MS) and selected ion flow tube mass spectrometry (SIFT-MS), were used to analyse the headspace of the main fungal species. Agar samples for *T. rubrum, T. mentagrophytes* and *M. canis* were prepared as illustrated earlier with three replicates each and uninoculated agar plates were used as controls. The sampling methodology for both the techniques is described below.

**a) SIFT-MS procedure**

For headspace sampling Nalophan bags were used that were constructed by using Nalophan tubing (Kalle UK Ltd.), with a filled diameter of 135mm. These may be sealed at one end by folding over and securing with a plastic tie after addition of the sample. The other end was attached around an inert plastic tube with a ¼ inch Swagelok fitting for connection to the SIFT-MS instrument inlet or a pump. These empty bags were then sterilised by UV irradiation for 24-72 hours. One agar plate (without its lid) was placed in each bag. The bag ends were sealed and filled with BOC zero grade (hydrocarbon free) air. A bag with only zero grade air was used as an additional control. These were incubated at 25°C for 96 hours for headspace generation. The bags were then heated to about 37-40°C for 5-10 minutes to allow for volatile compounds in the headspace to be increased in concentration through desorption from the Nalophan as well as reducing the volatiles' solubility in the media (Henry's Law). The bag fitting was then connected through the wall of the incubator, to the inlet capillary of the SIFT-MS instrument for analysis. The reaction of each SIFT-MS precursor ion ($H_3O^+$, $NO^+$ and $O_2^+$) with the sample was monitored for 90 seconds to generate mass spectra at mass/charge (m/z) values between 10 and 160 using the full scan mode.

**b) GC-MS procedure**

Automated thermal desorption (ATD) tubes were then connected to the bags (via an automated flow-controlled pump), filled with a headspace volume of 500 ml per tube and used for subsequent analysis by GC-MS. This method helps to preconcentrate the headspace. Standard stainless-steel ATDsorbent cartridges, containing dual packing comprising 50% Tenax TA and 50% Carbotrap (Markes International Limited, Llantrisant, UK) were used following conditioning[**]. Conditioned cartridges were sealed with locking caps and stored at 4ºC until required for use.

Captured volatiles were analysed using an AutoSystem XL gas chromatograph equipped with an ATD 400 thermal desorption system and TurboMass mass spectrometer (Perkin Elmer, Wellesley, MA). CP grade helium (BOC gases, Guildford, UK) was used as the carrier gas throughout. Cartridges were desorbed by purging for 2 min at ambient temperature then for 5 min at 300ºC. Volatiles purged from the cartridge were captured on a cold trap which was initially maintained at 30ºC. Once desorption of the cartridge was complete, the trap was heated to 320ºC using the fastest available heating rate and maintained at that temperature for 5 min whilst the effluent was transferred to the gas chromatograph via a heated (180ºC) transfer line coupled directly to the chromatographic column.

A Zebron ZB624 chromatographic column was used (Phenomenex, Torrance, CA). This is a wall-coated open tubular column (dimensions 30m×0.4mm×0.25mm ID), the liquid phase comprising a 0.25 μm layer of 6% cyanopropylphenyl and 94% methylpolysiloxane. The gas chromatograph oven was maintained at 50ºC for 4 min

[**] Conditioning was done by purging with He carrier gas for 2 min at 25°C followed by 30 min at 335°C.

following injection and was then raised at 10ºC $min^{-1}$ to 220ºC for 9 min. Separated products were transferred by a heated line to the mass spectrometer and ionised by electron bombardment. The spectrometer was set to carry out a full scan from m/z ratios 33 to 350 using a scan time of 0.3s with a 0.1s scan delay. The resulting mass spectra were combined to form a total ion chromatogram (TIC) by the GC-MS integral software (TurboMass 4.1).

### 2.2.6 Electronic nose systems

Two electronic nose systems were used for analysis; one comprising of an array of 14 conducting polymer (CP) sensors (BH114, Bloodhound Sensors, UK) and the other consisting of an array of 10 MOSFET sensors, 12 MOS sensors, together with an IR-based $CO_2$ sensor and a capacitance based Humidity sensor (NST 3320, Applied Sensor, Sweden), (Plate 2.1).

**Plate 2.1:** Left – BH114, Bloodhound; Right – NST Senstool, Nordic Technologies.

#### a) The Bloodhound (BH114)

Individual petri-plate cultures were placed in sampling bags (500 ml capacity, BDH), with the lid carefully removed, inflated with a fixed volume of filter-sterilized air and sealed. The bags were incubated for one hour at 25°C to allow equilibration for

headspace generation. Subsequently, the headspace from each bag was sampled through an air-filter system, which consisted of a needle attached to a bio-filter (0.45 µm, PTFE Whatman, HepaVent) and an activated carbon filter (Whatman), to ensure clean airflow. Samples were analysed in a random order, including the controls which consisted of blank agar plates (Keshri *et al.*, 1998; Keshri *et al.*, 2002; Needham *et al.*, 2005).

Alternatively, Universal bottles containing agar plugs were sealed with parafilm, covered with a cap and left to equilibrate as before. Headspace analysis was also carried out similarly.

The baseline for the system was set by passage of air through the activated carbon filter. In order to prevent carry over effects between samples, the array was flushed with filtered air again and allowed to return to its baseline, recovery stage (Figure 2.1).

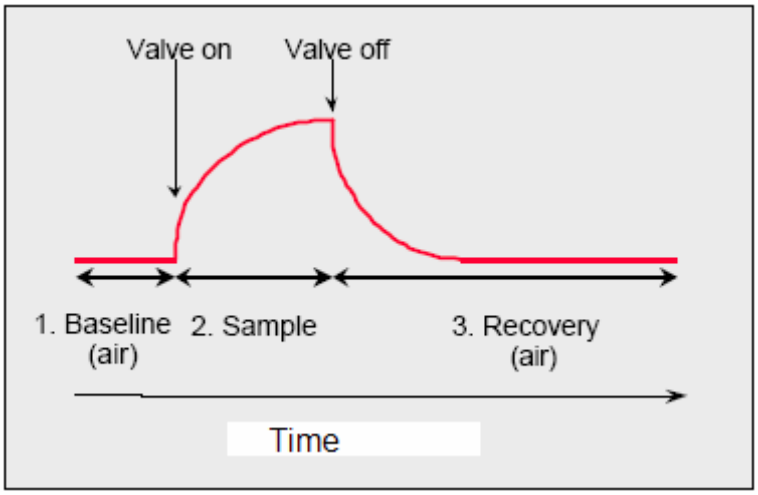



**Figure 2.1:** Measurement cycle

### b) The NST 3320

Sample vials were placed in the NST 3320 Lab Emission Analyser carousel (AS Nordic, Sweden) and randomly analysed. It employs an automated robotic needle set-up (described in Appendix B, B.1) to draw sample headspace from a sealed container

sampling vial, which is then exposed to the sensor array. Carry over effects were prevented in a similar manner as described earlier. The effect of drift on the sensors was tested over time by using certain controls (e.g. acetone, propan-1-ol, propan-2-ol, ethanol; figures in Appendix B, B.2).

### 2.2.7 Data analysis

The data collected was analysed by built-in software packages[††] in both electronic nose systems, Statistica 7 (Statsoft Inc.) and Matlab 7.2 (Mathworks Inc.). For the Bloodhound, normalised data for divergence, a sensor parameter indicating maximum step response, was analysed using XLStat (a Microsoft® Excel add-in). For the Nordic, the response parameter (mean-centred data) was chosen, which also indicated the maximum peak response for the various sensors.

Multivariate statistics involving Principal Component Analysis (PCA) and hierarchical Cluster Analysis (CA) were applied to the obtained sensor responses to check for discrimination between treatments. Loadings plots were also examined for the selection of suitable or relevant sensors. The results were displayed in the form of PCA scores plots and dendrograms in order to identify any possible relationships between the samples of the fungal species or the strains of the species. Additionally, a computational predictive model was built using the Matlab Neural Network Toolbox (v5) based on probabilistic neural networks (PNN), a type of radial basis network that would enable classification of the fungal samples into their respective classes i.e. species (details of which can be found in Appendix C).

The data generated by the GC-MS was analysed using the AMDIS (Automated Mass Spectral Deconvolution and Identification System) software. The chromatograms were

[††] There was no difference between the results obtained when compared with all software.

used to search the library contained in the NIST05 (National Institute of Standards and Technology) mass spectral database to identify compounds present in the fungal samples.

## *2.3 Results*

### 2.3.1 Comparison of electronic nose systems

The experiments performed initially had two objectives: one to determine the performance of the CP and hybrid sensor array systems and the other to differentiate three fungal species (*T. mentagrophytes, T. rubrum* and *T. verrucosum*) from each other based on their volatile production patterns.

Measurements using both electronic nose systems showed that after 24 hours incubation it was not possible to differentiate between any of the fungal species from the controls (uninoculated agar), when test species were grown on SABHI agar. There was practically no discrimination between the three fungal species and the blanks even after 72 hours using the CP system (Figure 2.2). On the other hand, with the Nordic system (metal oxide-metal ion sensor array) some differentiation after 48 hours and better discrimination between two species was obtained after about 72 hours incubation (Figure 2.3). The control agar treatment could not be distinguished from the third species examined (*T. verrucosum*).

The experiment was repeated with both the sensor array systems, but using an alternative sampling system for the Bloodhound where Universal bottles were used instead of sample bags to mimic the Nordic. Yet again, it did not appear to distinguish between the three fungi or controls even after 72 hours (Figure 2.4). This indicated that the hybrid sensor array based electronic nose produced better results.

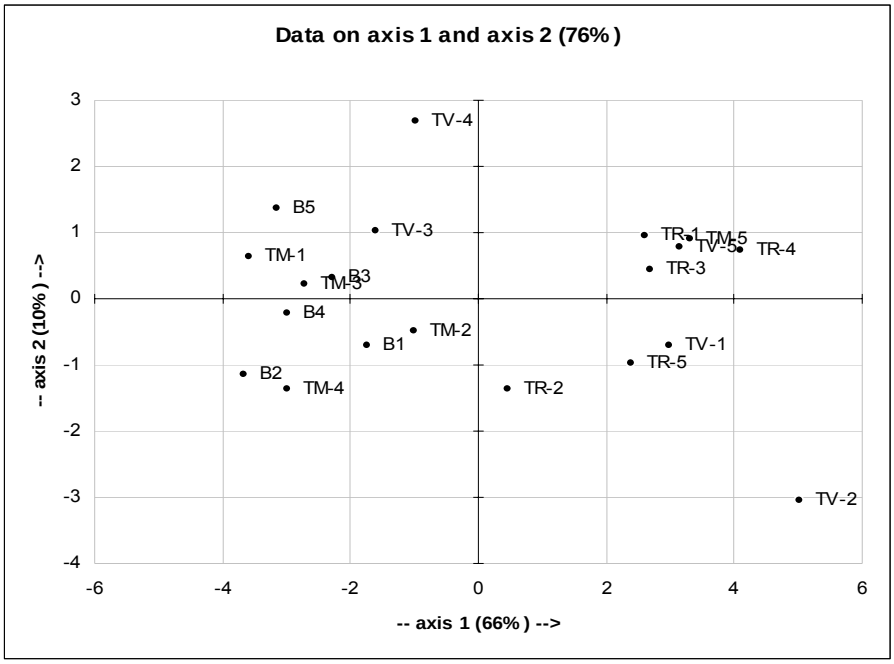



**Figure 2.2:** PCA plot after 72 hours at 25°C using the Bloodhound, showing no differentiation between the fungal species.
(*Key:* B – blank agar; TM – *T. mentagrophytes*; TR – *T. rubrum*; TV – *T. verrucosum*)

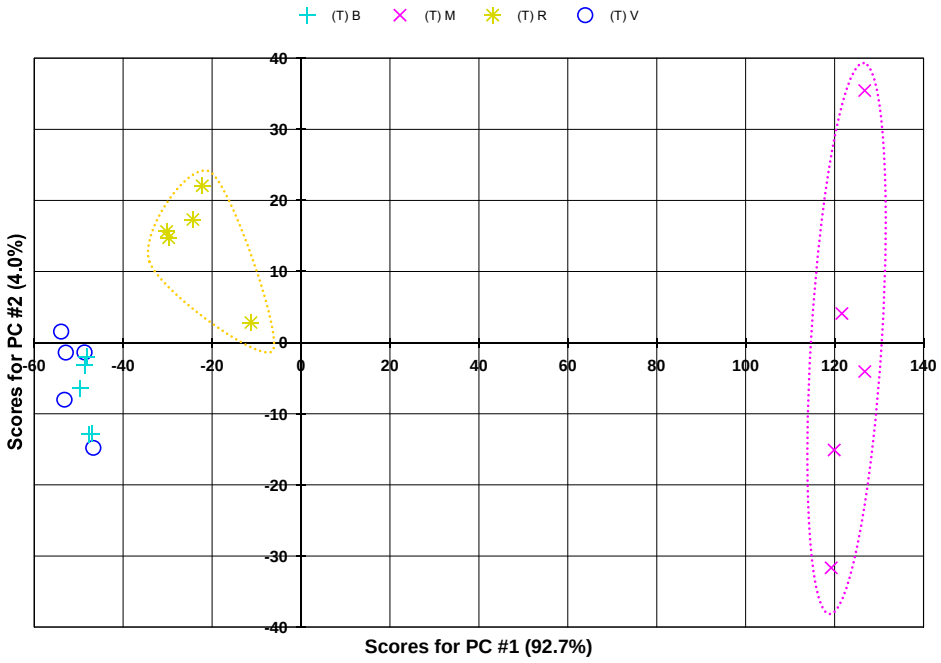



**Figure 2.3:** PCA map differentiating between *T. mentagrophytes* and *T. rubrum* after 72 hours at 25°C using the Nordic, self encircled.
(*Key:* B – blank agar; M – *T. mentagrophytes*; R – *T. rubrum*; V – *T. verrucosum*)

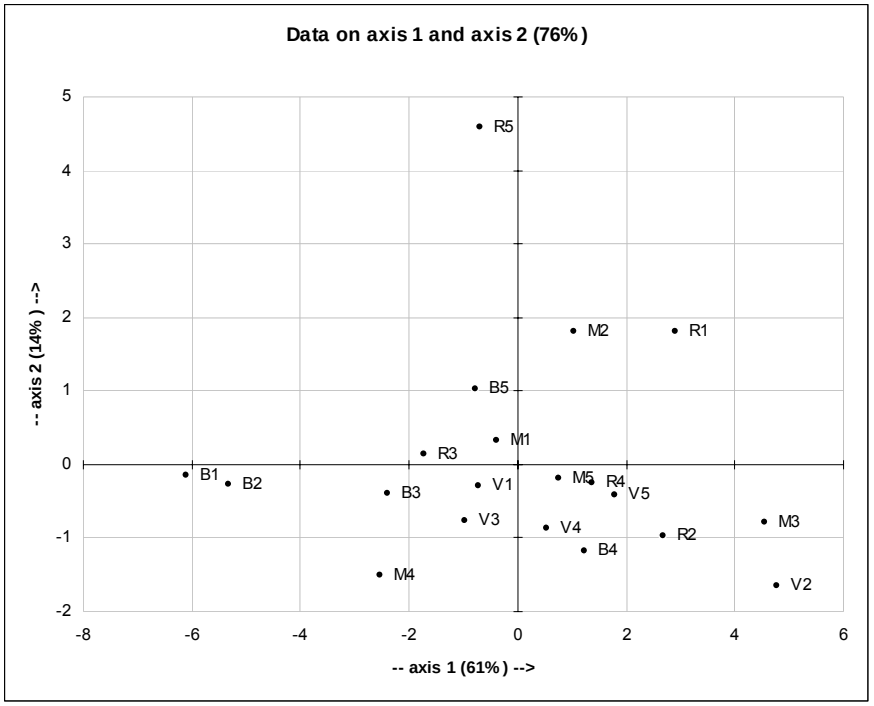



**Figure 2.4:** PCA plot after 72 hours at 25°C using an alternative sampling method with the Bloodhound depicting no distinction between samples.
(*Key:* B – blank agar; M – *T. mentagrophytes*; R – *T. rubrum*; V – *T. verrucosum*)

## 2.3.2 Intra-species (*Trichophyton*) discrimination

### a) Solid media based studies

The reproducibility of the sensors for the five replicates of an individual treatment and the controls using the Nordic electronic nose with hybrid sensors is illustrated in the form of two line graphs (Figures 2.5 and 2.6).

Since the PCA loadings plot indicated not all the sensor responses contributed to the potential for discrimination between species, only the relevant sensors were used for further analysis. Normally those variables away from the origin are more discriminating while those closer to the origin contribute to noise or have little or no information. An example is shown in Figure 2.7. By selecting only those variables that prove to be more discriminatory, the quality of differentiation can be improved (Figure 2.8).

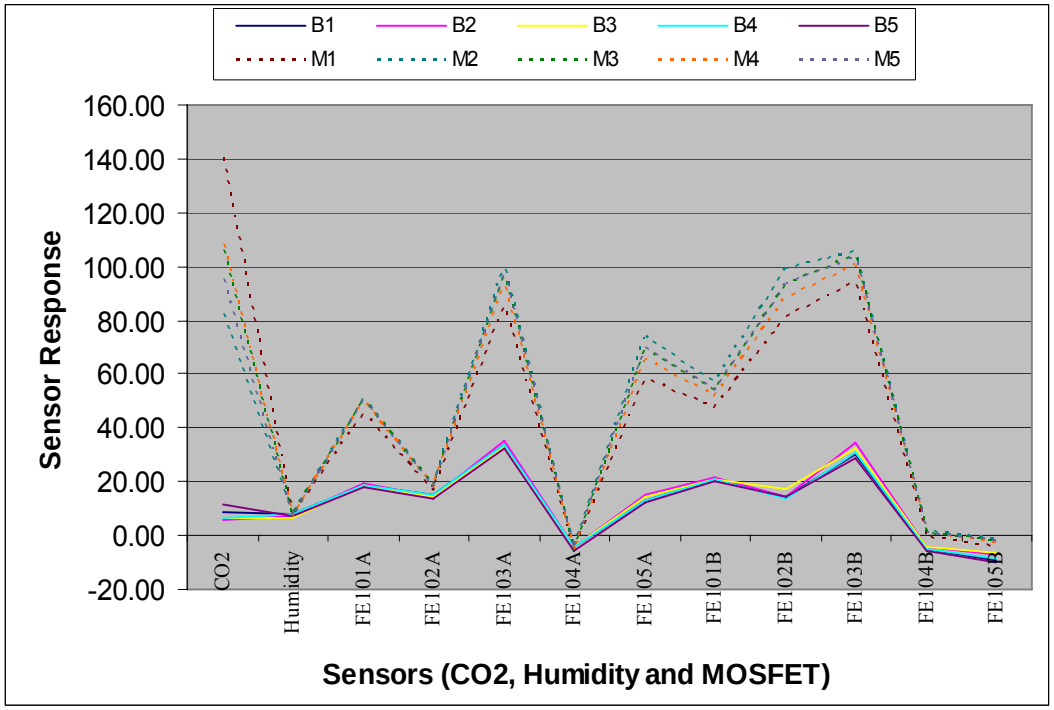



**Figure 2.5:** Signal parameters of one set of sensors from the Nordic for control (B) and *T. mentagrophytes* (M) after 72 hours showing sensor reproducibility.

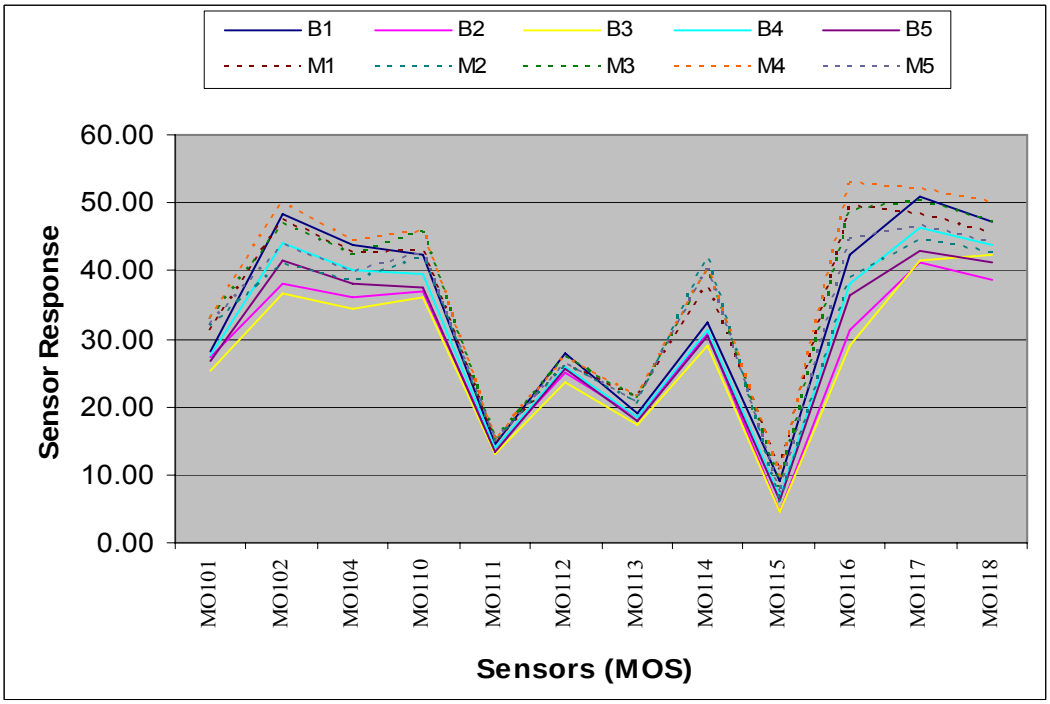



**Figure 2.6:** Reproducibility of MOS sensors in the Nordic for control (B) and *T. mentagrophytes* (M) after 72 hours.

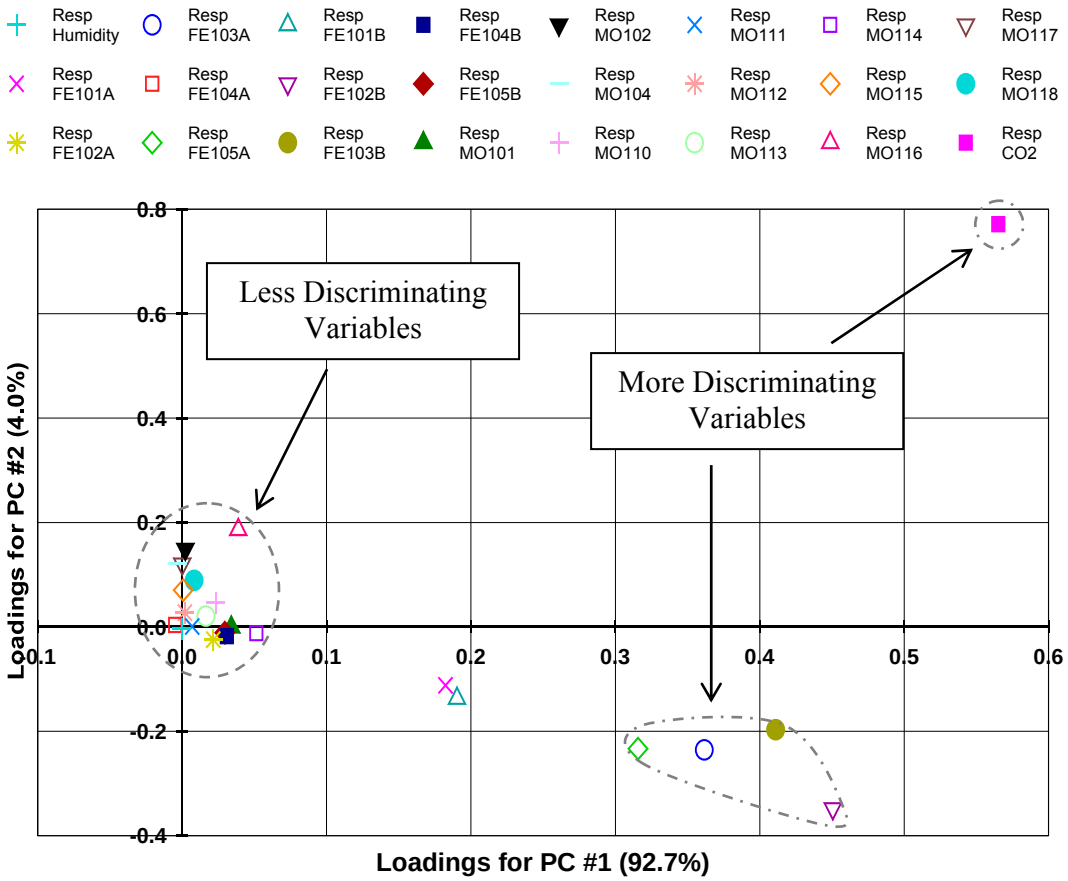



**Figure 2.7:** PCA loadings plot after 72 hours growth on SABHI agar at 25°C using the Nordic (data from figure 2.3)

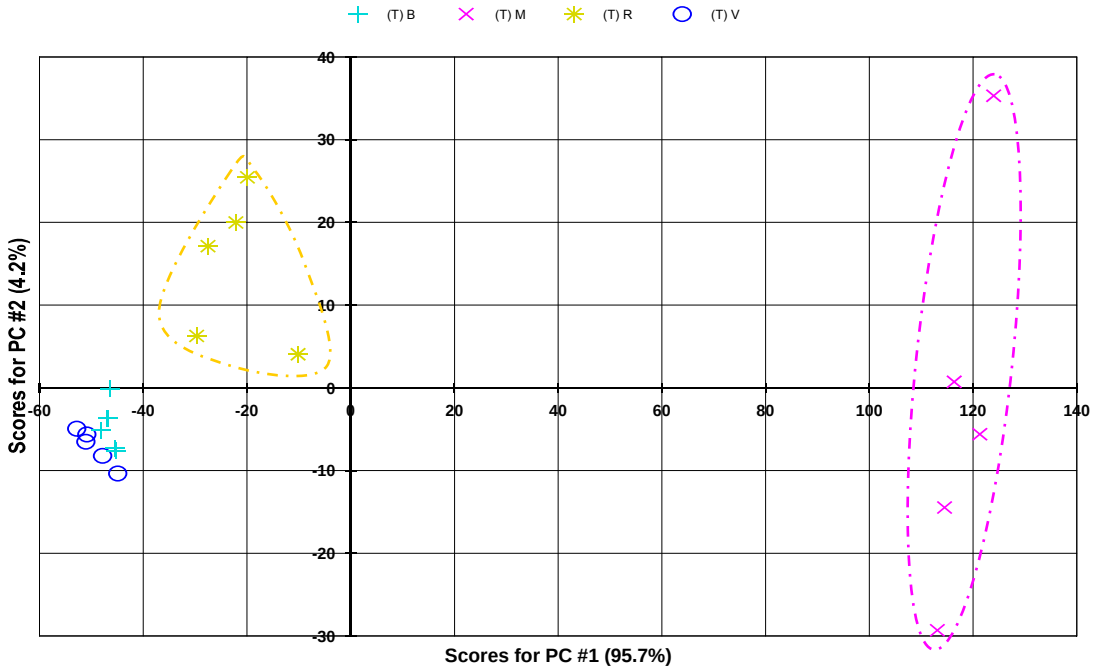



**Figure 2.8:** PCA scores plot after selecting sensors indicated in the previous figure. (*Key:* B – blank agar; M – *T. mentagrophytes*; R – *T. rubrum*; V – *T. verrucosum*)

On comparing Figures 2.3 and 2.8, it can be seen that the variance captured in the latter is greater, approximately 99.9% of the data, which implies that by selecting the most important sensors more information can be retained with little loss. When the same data was analysed using hierarchical cluster analysis (with Euclidean distance to measure distances between samples forming clusters and Ward's linkage method to establish the distance amid the clusters), it could be seen that *T. mentagrophytes* and *T. rubrum* formed two distinct clusters; whereas the controls and the third species (*T. verrucosum*) appeared to be grouped together (Figure 2.9).

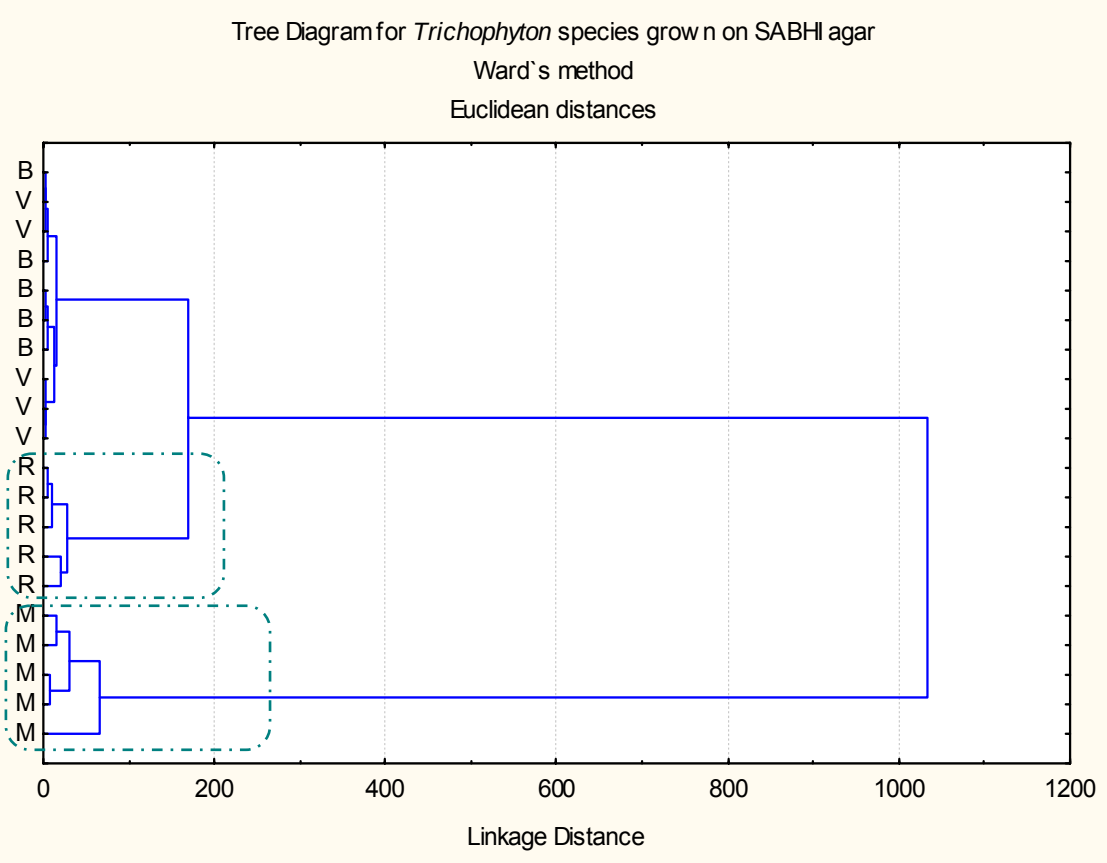



**Figure 2.9:** Cluster analysis of the *Trichophyton* species after 72 hours at 25°C.

(*Key:* B – blank agar; M – *T. mentagrophytes*; R – *T. rubrum*; V – *T. verrucosum*)

Similar experiments were also performed on another medium, SDA, commonly used in isolating dermatophytes from clinical samples. The results were comparable to the earlier experiment; where after 96 hours growth two of the dermatophyte species could be distinguished whilst *T. verrucosum* and the controls could not (data not shown). Figure 2.10 illustrates the mean $CO_2$ response over time for the different treatments. This shows clearly that the growth of the species varies with *T. mentagrophytes* and *T. rubrum* growing much faster than the *T. verrucosum*.

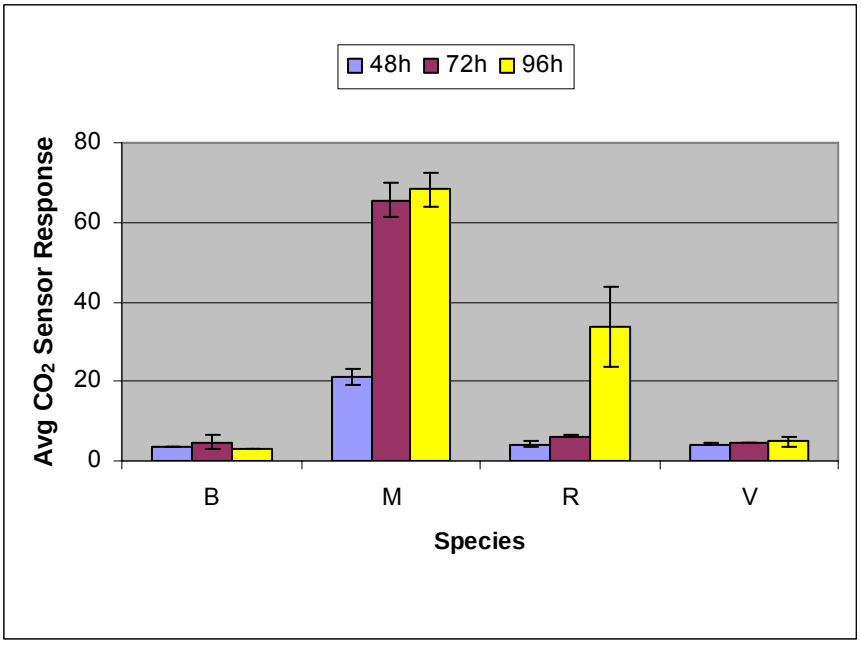



**Figure 2.10:** Average $CO_2$ production over time after growth on SDA at 25°C for the fungal species.

(*Key:* B – blank agar; M – *T. mentagrophytes*; R – *T. rubrum*; V – *T. verrucosum*; I – standard error bars)

Subsequent experiments comprised all four dermatophyte species which included a new strain of *T. rubrum* where analysis after 72 hours separated *T. mentagrophytes*; but PCA analysis after 96 hours showed five distinct clusters segregating the four fungal species

and the controls. One sample of *T. verrucosum* and *T. violaceum* however fell into different groups (highlighted) as shown in Figure 2.11. Considering that these might be outliers, they were removed and based on the loadings plot five sensors were selected. This improved the variance captured from 89.7% to about 93.4% (Figure 2.12). The formation of five distinct clusters was further corroborated by cluster analysis by means of Ward's linkage and Euclidean distance, whereby each species was grouped tightly into its respective cluster (Figure 2.13).

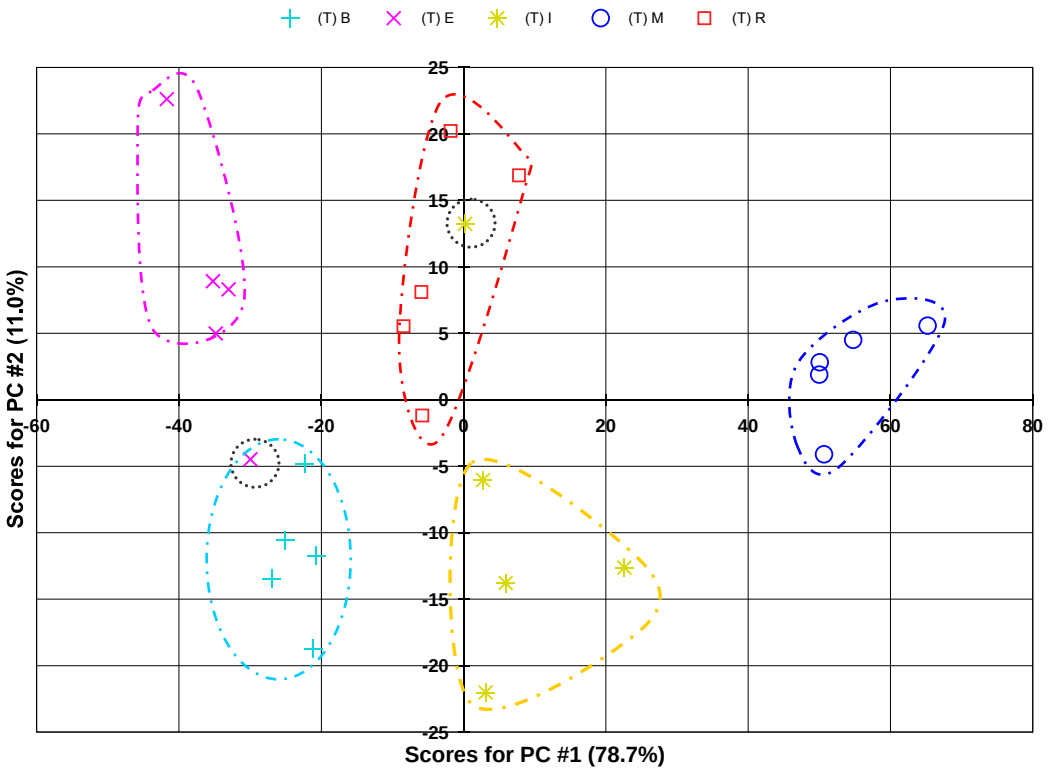



**Figure 2.11:** PCA scores plot showing five distinct clusters (self highlighted) after 96 hours growth on SDA at 25°C with grey circles indicating probable outliers.

(*Key:* B – blank agar; M – *T. mentagrophytes*; R – *T. rubrum*; E – *T. verrucosum*; I – *T. violaceum*)

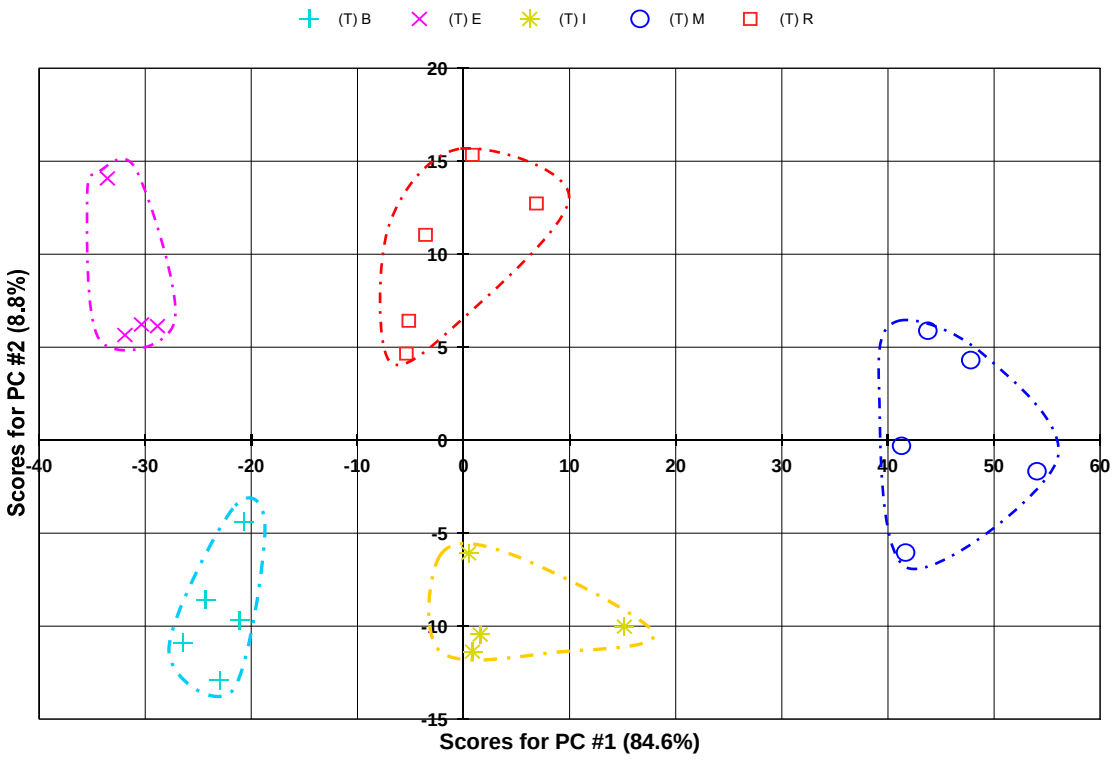



**Figure 2.12:** PCA scores plot illustrating clear discrimination and increased variance between the four fungal species and the blank agar within 96 hours at 25°C on SDA after outlier removal and suitable sensor selection.

(*Key:* B – blank agar; M – *T. mentagrophytes*; R – *T. rubrum*; E – *T. verrucosum*; I – *T. violaceum*)

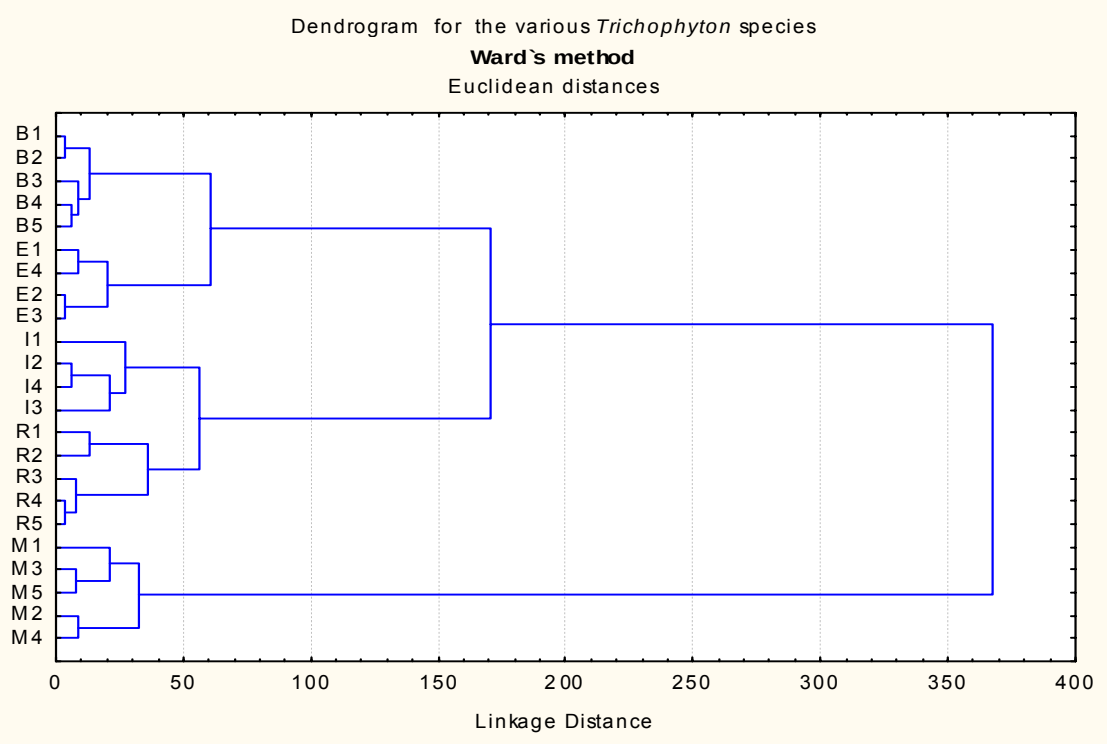



**Figure 2.13:** Tree diagram showing distinct clusters of the four fungal organisms after 96 hours growth on SDA at 25°C.

(*Key:* B – blank agar; M – *T. mentagrophytes*; R – *T. rubrum*; E – *T. verrucosum*; I – *T. violaceum*)

**b) Computational model for class prediction**

Probabilistic neural networks were created using several variations of the sensors involved using the e-nose data from the 96 hour growth of the four fungal species. The use of all sensors resulted in higher rates of misclassified samples as seen in the resulting confusion matrix in Table 2.1 ranging from 28 to 32%. However, by excluding certain sensors (e.g. humidity, FE104A, FE105A, FE102B, FE104B, FE105B) based on the PCA loadings, the classification rate improved and increased to almost 88 or 96% (Table 2.2). On comparison with Figure 2.11, one sample from *T. violaceum* and *T. verrucosum* each are misclassified using eighteen sensors, however a *T. rubrum* sample is also misclassified. Furthermore, the use of only five sensors results in only one misclassified sample but it could imply overfitting.

**Table 2.1:** Confusion matrix indicating the classification accuracy for each of the samples using all sensors, 28% of samples misclassified.

(*Key:* B – blanks; E – *T. verrucosum*; I – *T. violaceum*; M- *T. mentagrophytes*; R- *T. rubrum*)

| | | Predicted Class | | | | |
|---|---|---|---|---|---|---|
| | | B | E | I | M | R |
| Actual Class | B | 5 | 0 | 0 | 0 | 0 |
| | E | 1 | 3 | 0 | 1 | 0 |
| | I | 0 | 0 | 4 | 0 | 1 |
| | M | 0 | 0 | 0 | 5 | 0 |
| | R | 2 | 2 | 0 | 0 | 1 |

**Table 2.2:** Confusion matrices based on the input of data from either 18 sensors (A) or five sensors (B) with prediction accuracy of 88% and 96% respectively.

(*Key:* B – blanks; E – *T. verrucosum*; I – *T. violaceum*; M- *T. mentagrophytes*; R- *T. rubrum*)

| (A) | Predicted Class | | | | |
|---|---|---|---|---|---|
| | B | E | I | M | R |
| B | 5 | 0 | 0 | 0 | 0 |
| E | 1 | 4 | 0 | 0 | 0 |
| I | 0 | 0 | 4 | 0 | 1 |
| M | 0 | 0 | 0 | 5 | 0 |
| R | 1 | 0 | 0 | 0 | 4 |

| (B) | Predicted Class | | | | |
|---|---|---|---|---|---|
| Actual Class | B | E | I | M | R |
| B | 5 | 0 | 0 | 0 | 0 |
| E | 1 | 4 | 0 | 0 | 0 |
| I | 0 | 0 | 5 | 0 | 0 |
| M | 0 | 0 | 0 | 5 | 0 |
| R | 0 | 0 | 0 | 0 | 5 |

The effect of the varying 'spread constant' values was not only seen to affect the accuracy of the network to predict the samples into their respective classes but also varied with the number of sensors used. In case of fewer sensors, spread values below 0.5 had a tendency for better classification (Figure 2.14). The performance of the network was then evaluated by means of regression analysis shown in Figure 2.15. This indicated a good fit as the points were in close proximity to the line of perfect fit (dashed line) including a relatively high correlation coefficient.

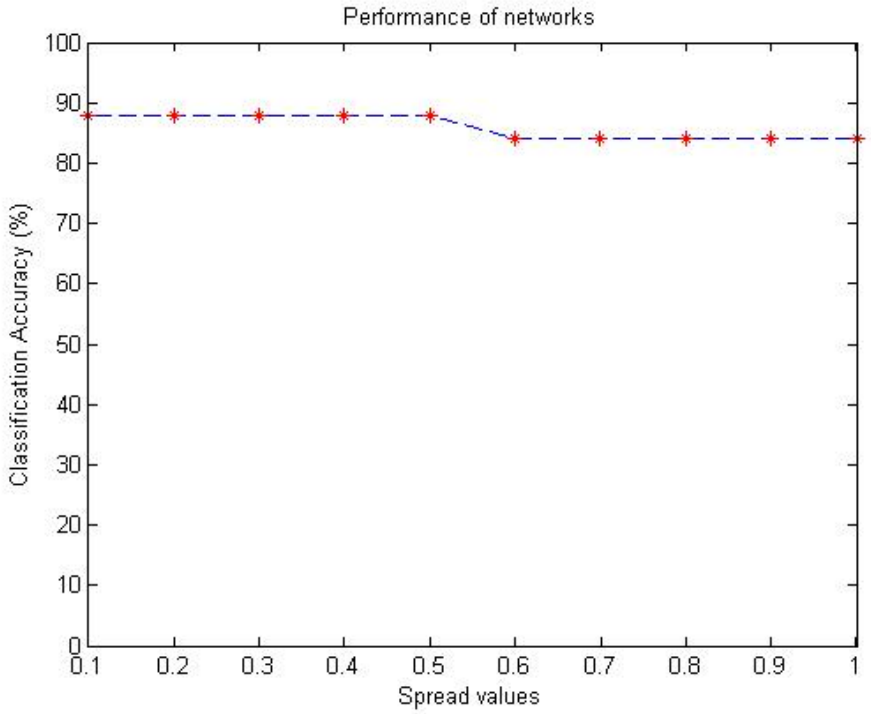



**Figure 2.14:** Networks classification ability based on different spread constant values using data from 18 sensors.

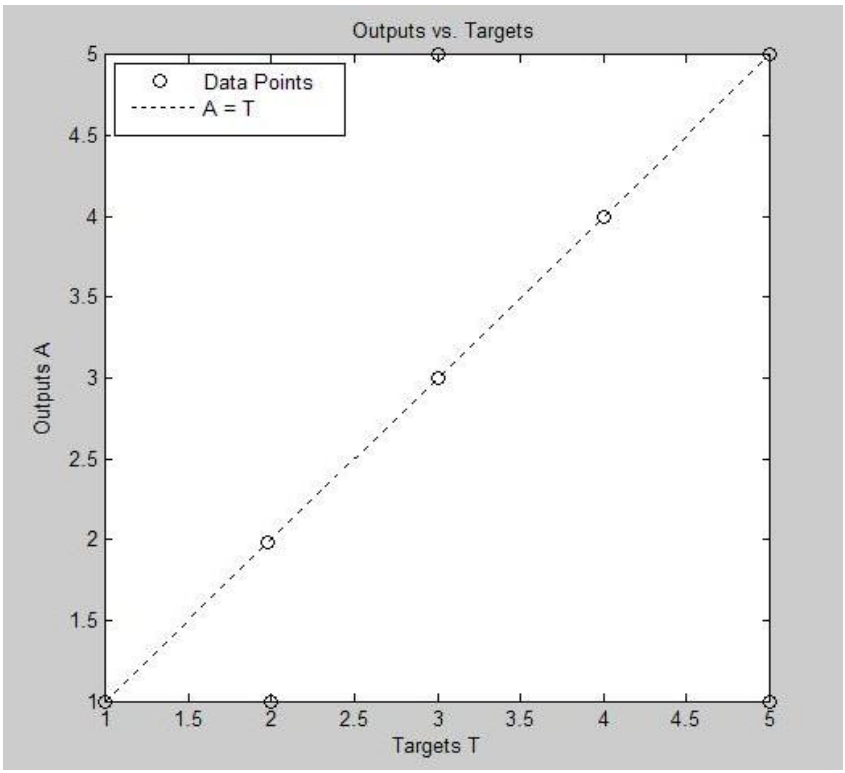



**Figure 2.15:** Comparison between the actual and predicted outputs based on spread of 0.5.

### c) Liquid culture studies

Experiments were also conducted in liquid broth in order to discriminate between the two faster growing species, i.e. *T. mentagrophytes* and *T. rubrum,* from un-inoculated broth controls. Sampling and analysis every 24 hours was performed for 120 hours. In the PCA after 48 and 72 hours respectively, only *T. mentagrophytes* could be distinguished. Although the control and *T. rubrum* appeared to be separated, cluster analysis showed otherwise (Figure 2.16). Further studies are required to optimise the use of liquid medium.

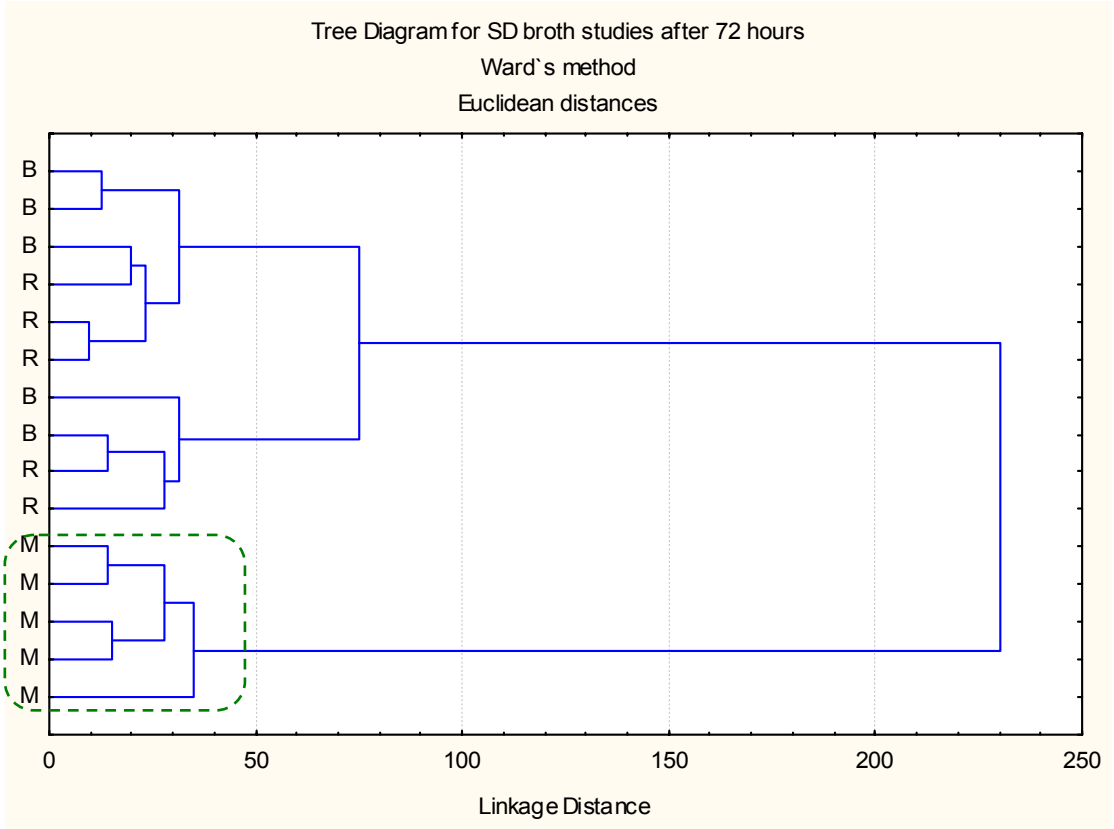



**Figure 2.16:** Liquid broth studies showing only one organism to be separated by means of a dendrogram after 72 hours at 25°C.
(*Key:* B – blank; M – *T. mentagrophytes*; R – *T. rubrum*)

### 2.3.3 Sensitivity thresholds for detection

The main purpose of these experiments was to try to identify the threshold concentrations at which the fungal spores of *T. mentagrophytes* and *T. rubrum* could be detected by means of volatile fingerprinting. The treatments used were in the range of log1, log3, log5 and log7 (i.e. from $10^1$ to $10^7$) CFUs $ml^{-1}$. On SABHI agar, within 72 hours log5 and log7 could be differentiated (data not shown). However, after 96 hours it was possible to discriminate between three treatments: log3, log5 and log7 as illustrated in Figure 2.17. This accounted for roughly over 99% of the data with just the first two principal components. This was confirmed by cluster analysis where three distinct clusters were observed as seen in Figure 2.18. The increasing trend of sensitivity detection over time could also be observed in the mean $CO_2$ production shown in Figure 2.19.

Studies on SDA also enabled separation between three treatments after 96 hours, with initial concentrations of log3, log5 and log7 CFUs $ml^{-1}$. Although the PCA scores plot showed clusters accounting for approximately 89% of the data, the dendrogram constructed did not produce tight clusters of log7 and log5 as before (Figure 2.20). Regardless of medium used, the control appeared to separate out only after 120 hours.

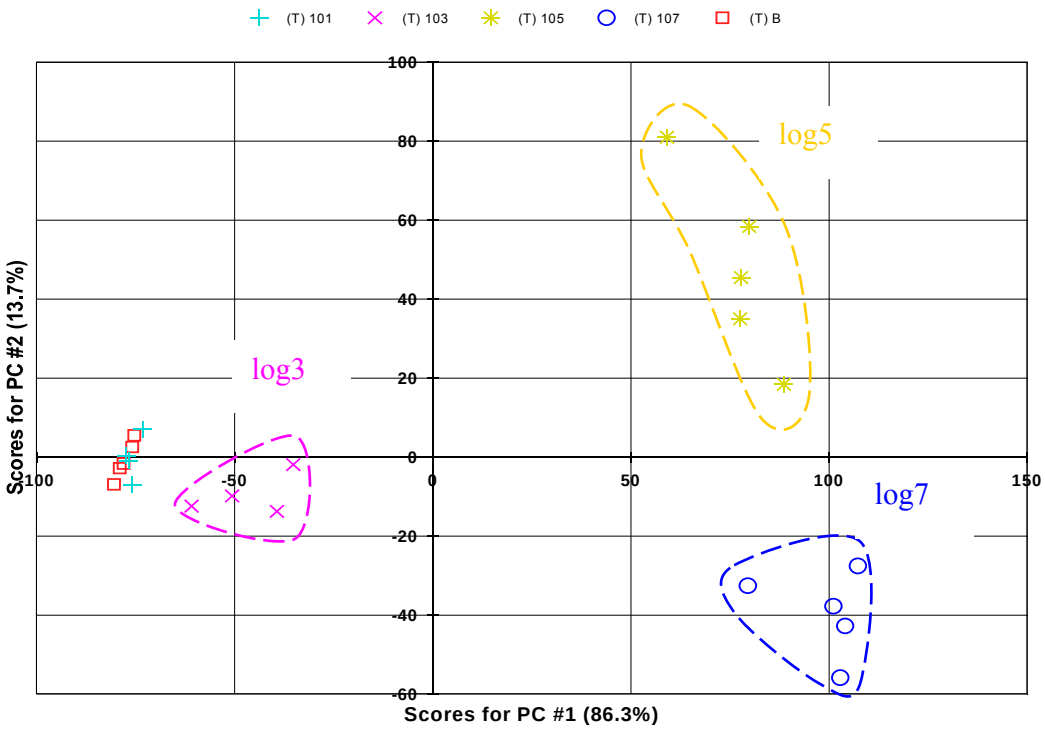



**Figure 2.17:** PCA scores plot showing three distinct groups within 96 hours at 25°C on SABHI agar for *T. mentagrophytes*.

(*Key:* B – blank; 101 – log1; 103 – log3; 105 – log5; 107 – log7)

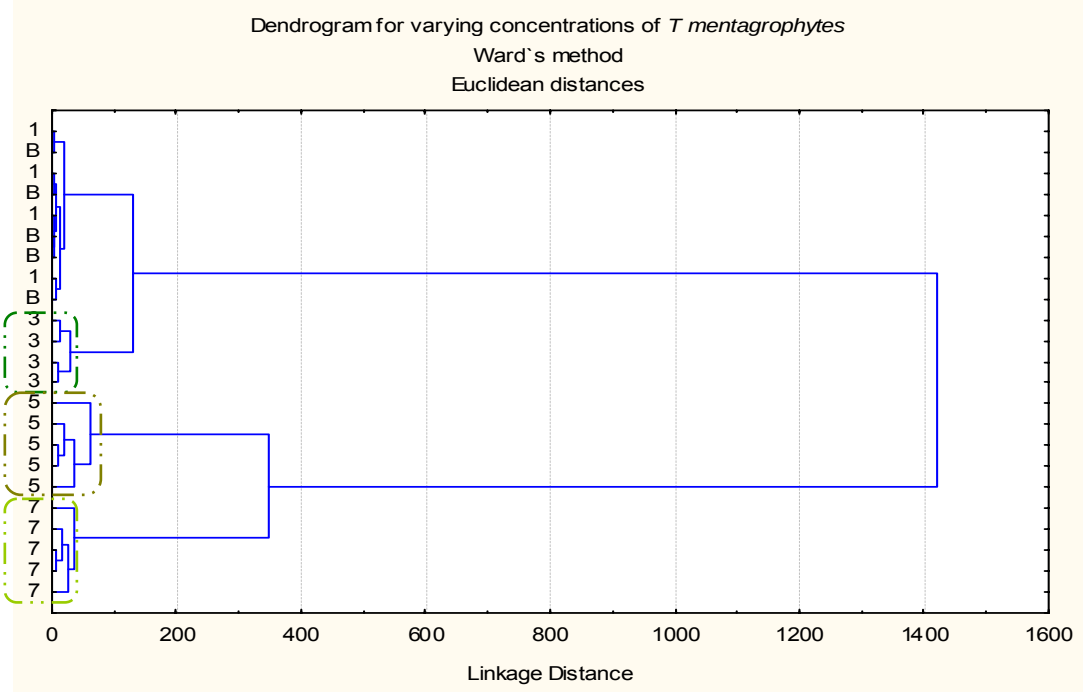



**Figure 2.18:** *T. mentagrophytes* showing three tight clusters after 96 hours at 25°C on SABHI agar.

(*Key:* B – blank; 1 – log1; 3 – log3; 5 – log5; 7 – log7)

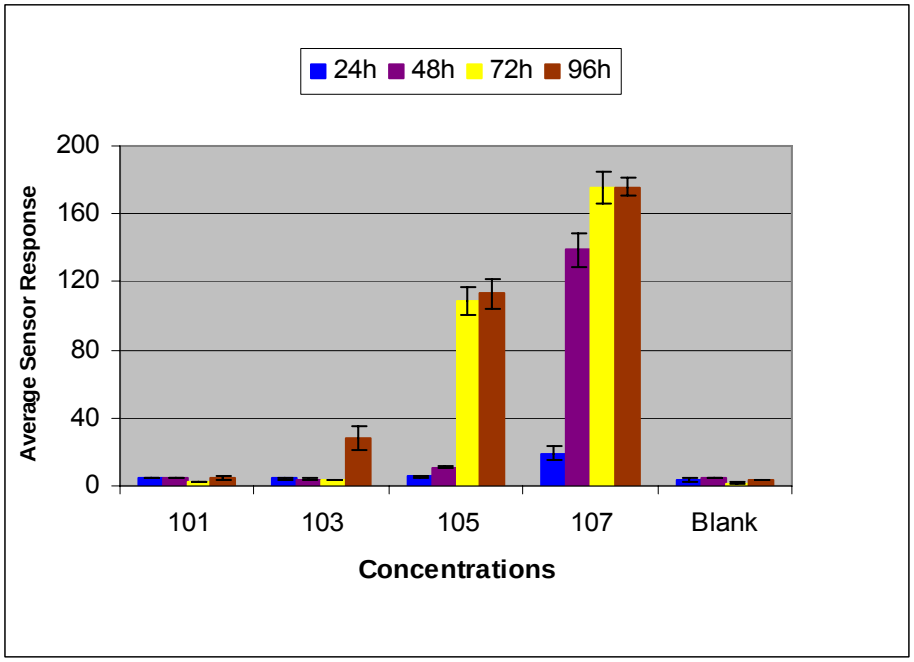



**Figure 2.19:** Mean temporal production of $CO_2$ for the different sensitivities of *T. mentagrophytes* grown on SABHI agar at 25°C.

(*Key:* 101 – log1; 103 – log3; 105 – log5; 107 – log7; I – standard error bars)

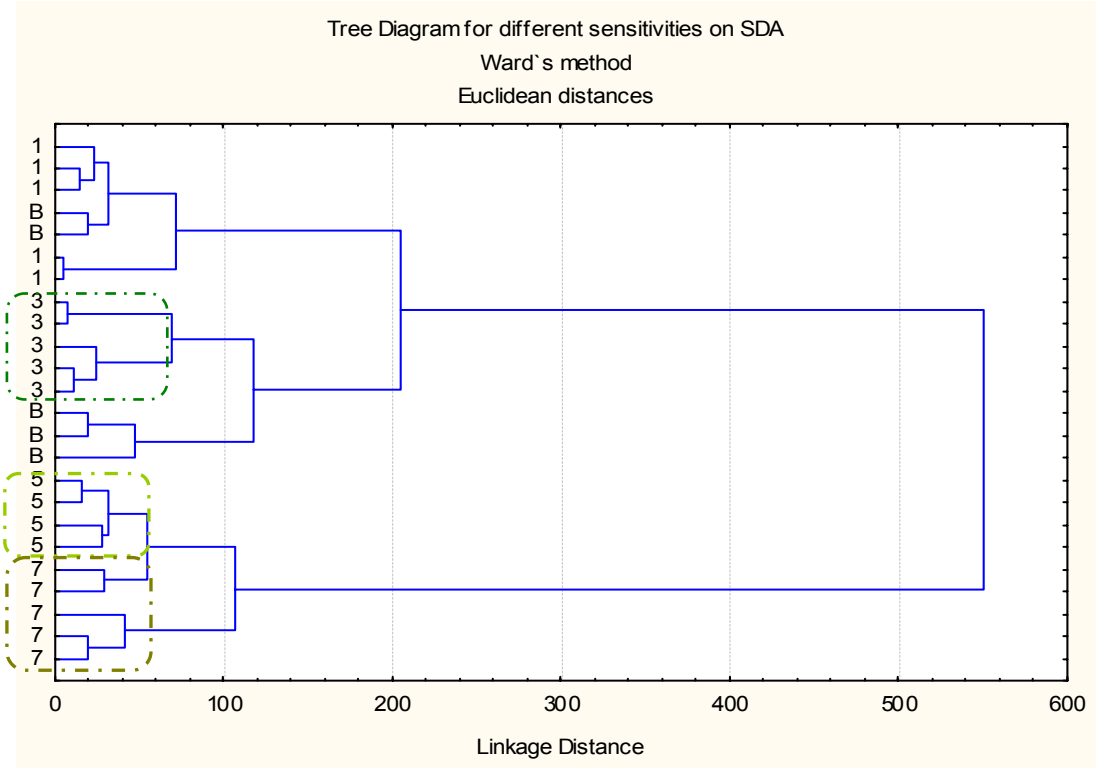



**Figure 2.20:** Dendrogram of the varying sensitivities of *T. mentagrophytes* after 96 hours growth on SDA at 25°C.

(*Key:* B – blank; 1 – log1; 3 – log3; 5 – log5; 7 – log7)

Experiments to detect the sensitivities in the range of log1, log3, log5 and log7 spores $ml^{-1}$ for *T. mentagrophytes* were also conducted in SD broth. In this study the initial concentrations of log3, log5 and log7 appeared to be distinguishable in about 72 hours as shown in Figure 2.21 after selecting suitable sensors, while the control and log1 treatments did not seem to separate until much later i.e. around 120 hours. Cluster analysis however indicated that the log3 replicates clustered together amidst a mixed grouping of blanks and log1 treatments (data not shown).

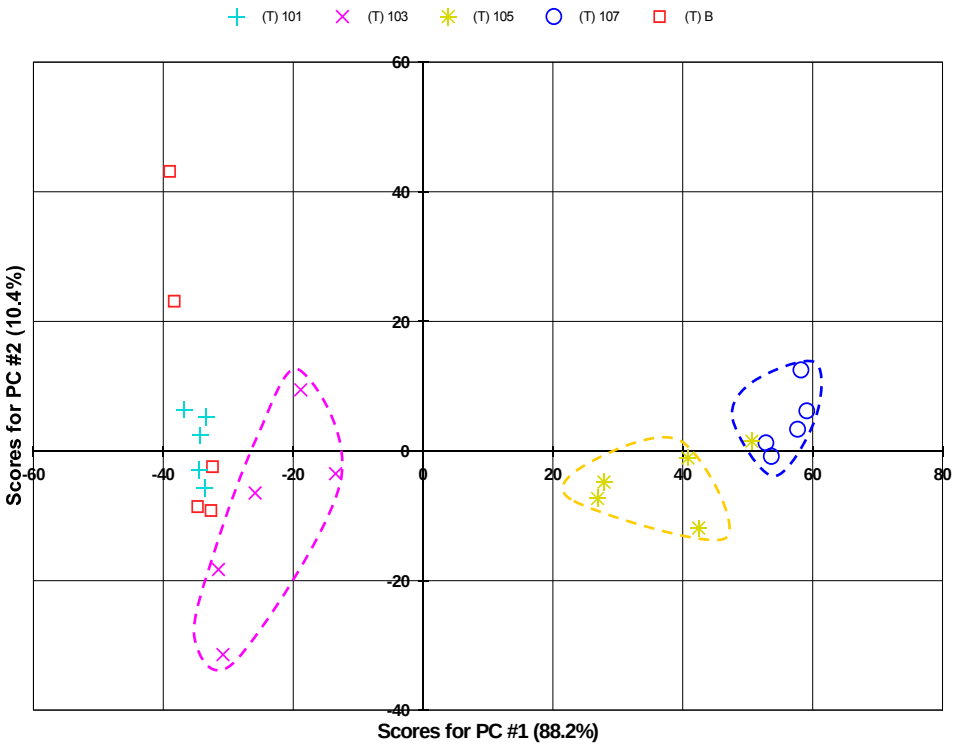



**Figure 2.21:** PCA scores plot differentiating three sensitivities of *T. mentagrophytes* in liquid broth at 25°C in about 72 hours.
(*Key:* B – blank; 101 – log1; 103 – log3; 105 – log5; 107 – log7)

Similar experiments were also performed with *T. rubrum* (original type strain) to detect the sensitivities in the range of log1, log3, log5 and log6 spores $ml^{-1}$ in SD broth. Within 96 hours it was possible to observe some discrimination between log6 and log5, with

the log6 replicates being quite spread out (Figure 2.22). On the other hand, more segregation was observed by omitting log6 treatments (log1, log3 and log5). Nevertheless, cluster analysis showed only log5 to be clustered whilst the other treatments had a mixed grouping (Figure 2.23). The respiratory growth pattern is shown in Figure 2.24 based on mean $CO_2$ production. There was consistency in this parameter during the experiment.

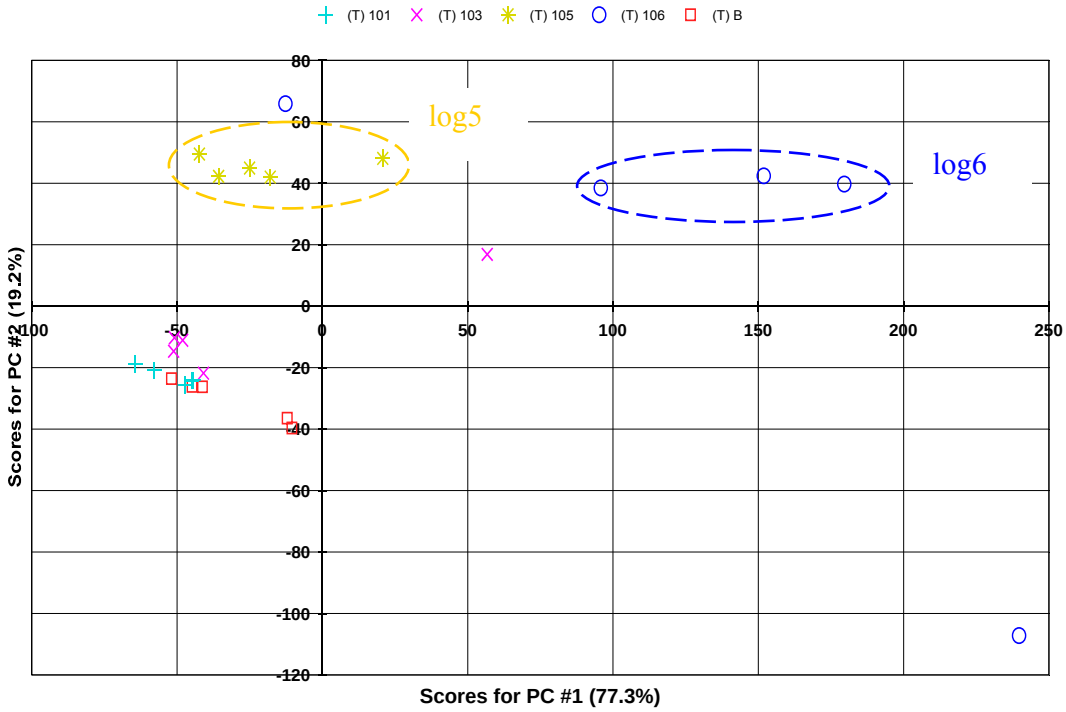



**Figure 2.22:** PCA plot of 96 hours of *T. rubrum* in liquid broth at 25°C showing different sensitivities.

(*Key:* B – blank; 101 – log1; 103 – log3; 105 – log5; 106 – log6)

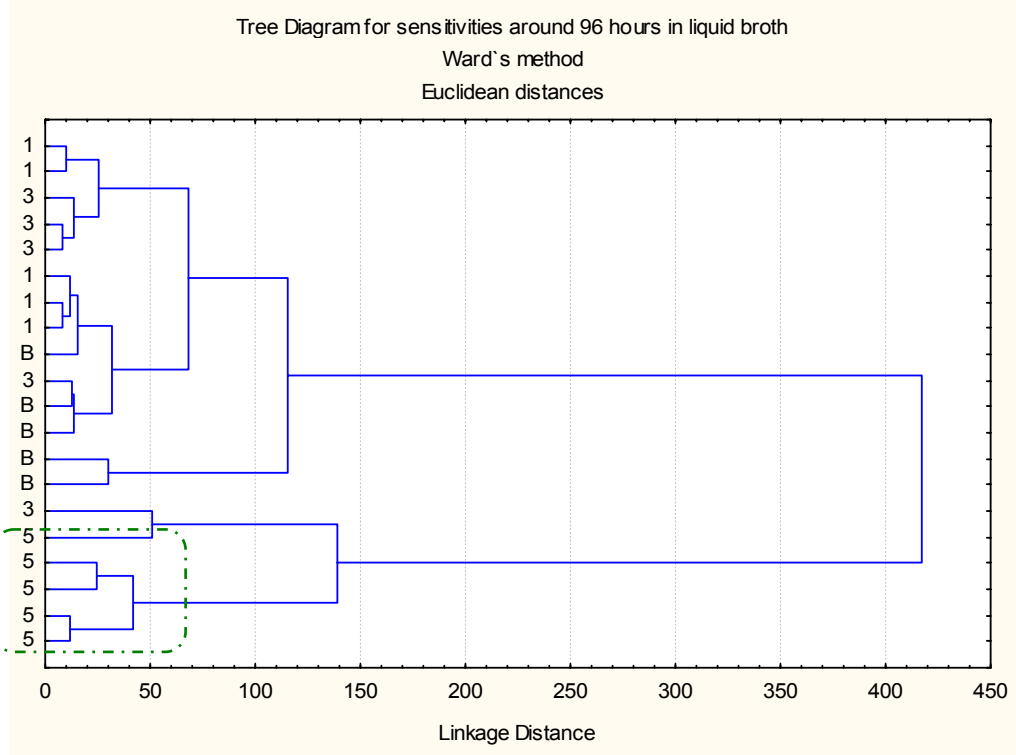



**Figure 2.23:** Dendrogram illustration with omitted log6 data for *T. rubrum* in broth at 25°C.

(*Key:* B – blank; 1 – log1; 3 – log3; 5 – log5)

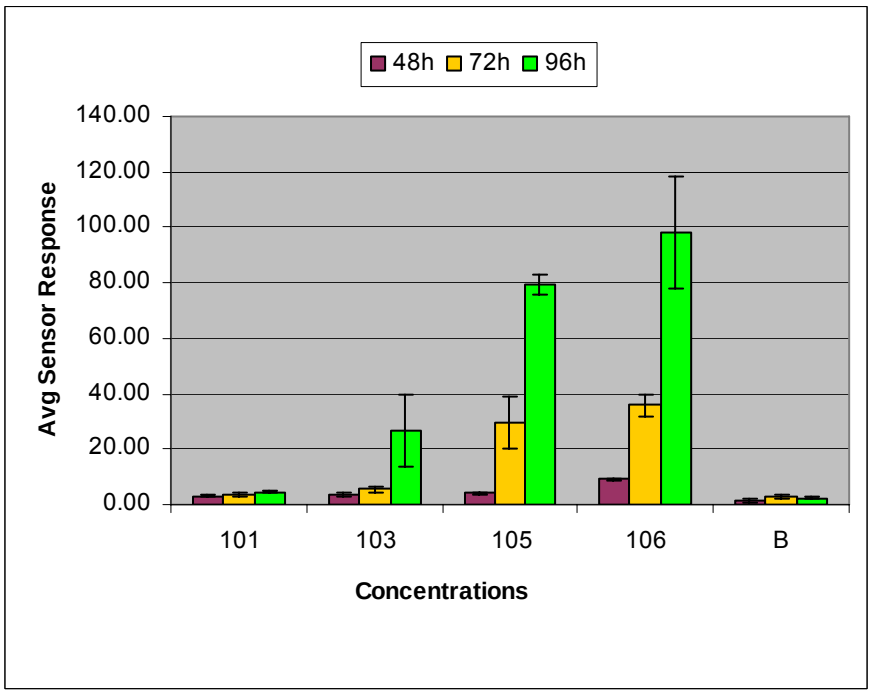



**Figure 2.24:** Average temporal $CO_2$ production for *T. rubrum* cultured in liquid broth at 25°C.

(*Key:* B – blank; 101 – log1; 103 – log3; 105 – log5; 106 – log6; I – standard error bars)

### 2.3.4 Intra-strain similarity/differences for *Trichophyton* species

Five strains of *T. mentagrophytes* and four strains of *T. rubrum* were examined to study the consistency of volatile profiles produced to detect similarities or differences amongst the strains. Results for *T. mentagrophytes* suggest that the controls can be separated from the strains/treatments after about 48 hours, but this was more clearly evident over 72 hours. However, even after 96-120 hours these strains could not be differentiated from each other (Figure 2.25).

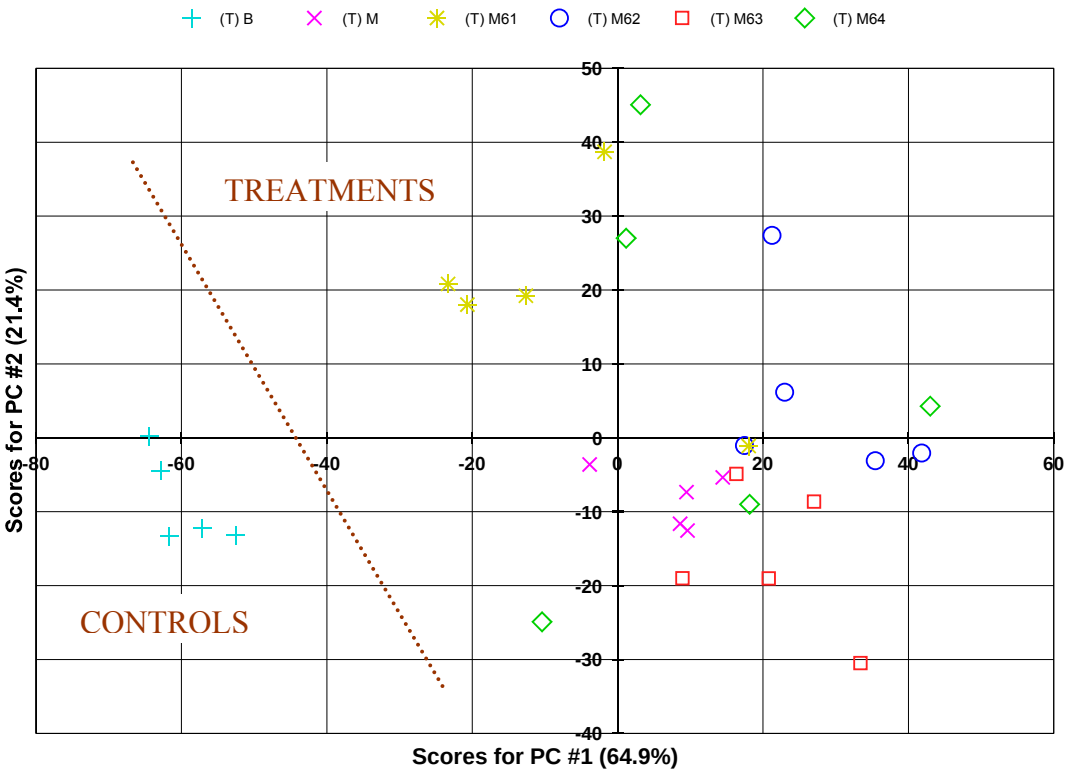



**Figure 2.25:** PCA scores plot after 96 hours on SDA at 25°C showing discrimination between controls (B) and *T. mentagrophytes* strains (M, M61, M62, M63, M64).

In contrast, experiments with *T. rubrum* indicated that within 120 hours it was possible to differentiate between controls and three of the strains of this fungal species – R, R55

and R57. The PCA scores plot in Figure 2.26 depicts the separation between the samples after 120 hours incubation. The data accounted for approximately 86% of the variance in the first two principal components. Subsequent cluster analysis on the data also showed that strains R, R55 and R57 could be clustered while the controls and R59 could not be effectively discriminated (Figure 2.27). However, a sample from R55 was shown to be clustered with R. The dendrogram was constructed as described previously.

These findings were substantiated when growth rates of these strains were observed, which indicated that strains of *T. mentagrophytes* grew at approx. the same rate whilst those of *T. rubrum* were variable – some growing slower than others (Figure 2.28)

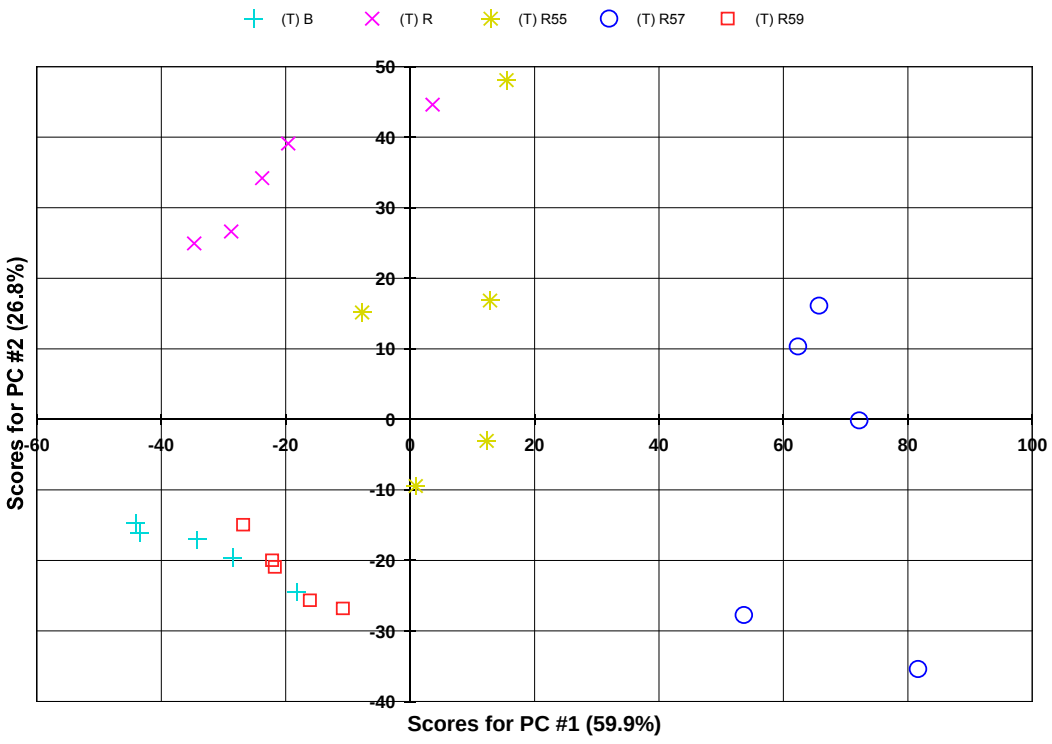



**Figure 2.26:** PCA scores plot depicting discrimination between 3 strains of *T. rubrum* (R, R55, R57) after 120 hours at 25°C on SDA from controls (B).

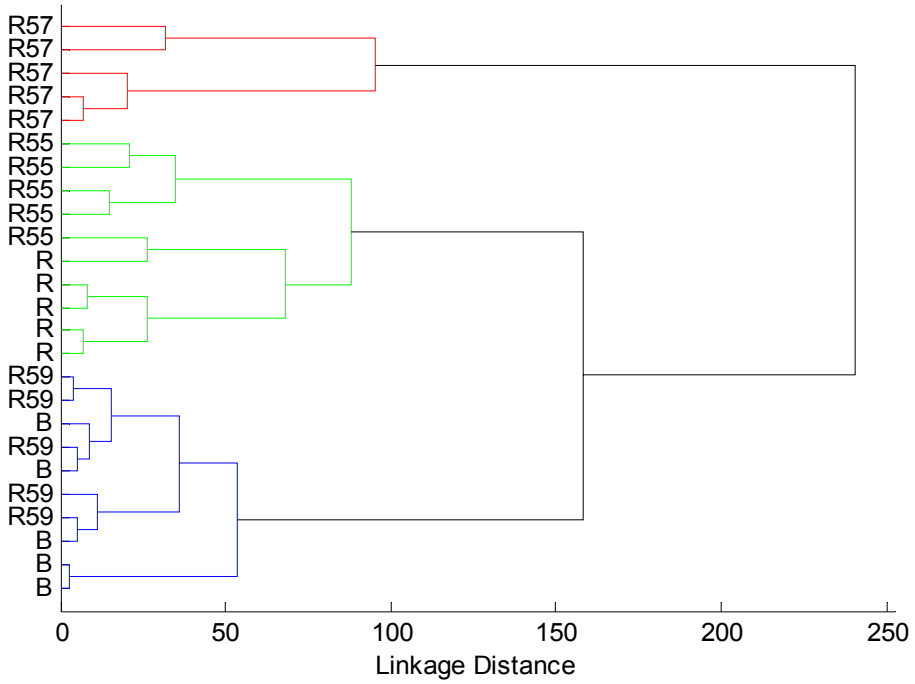



**Figure 2.27:** Dendrogram based on the first 2 PCs of fungal strains after 120 hours at 25°C on SDA.

(*Key:* B – Controls and R, R55, R57, R59 – *T. rubrum*)

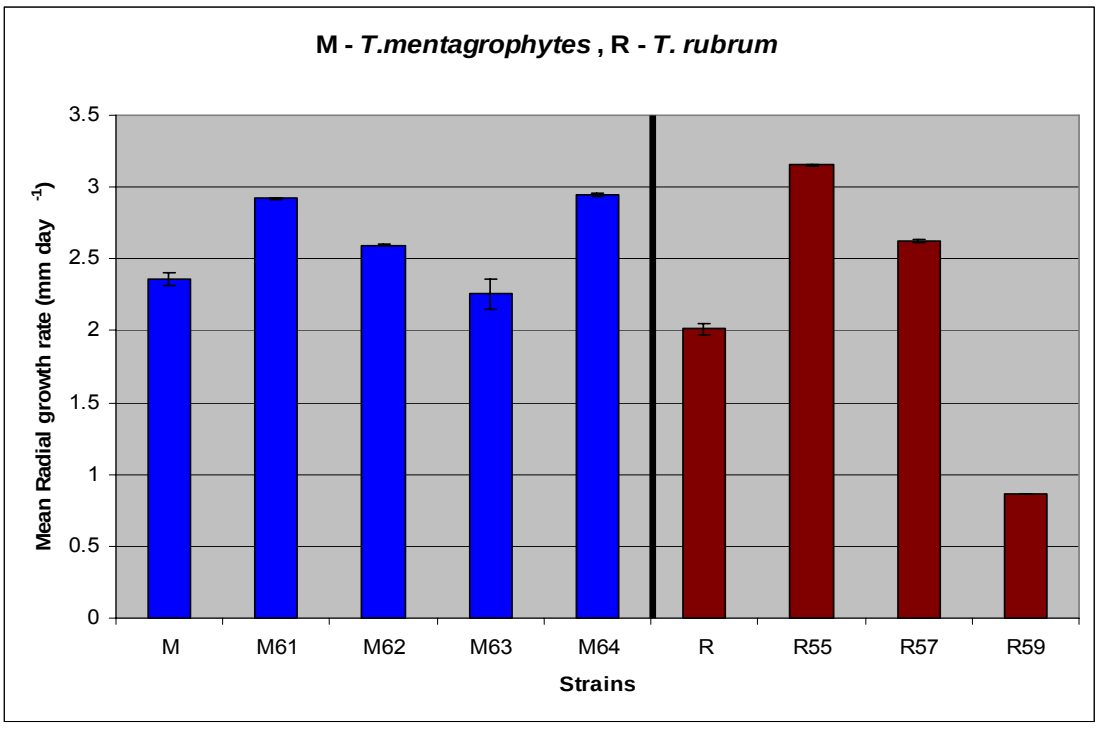



**Figure 2.28:** Growth rates of the strains of *T. mentagrophytes* and *T. rubrum* at 25°C on SDA. Bars indicate standard error of mean.

### 2.3.5 Inter-species discrimination

To investigate the potential of discriminating between dermatophytes causing human and animal infections *T. mentagrophytes, T. rubrum* and *M. canis* were studied for differences in their volatile fingerprint production patterns. Experiments indicated possible differentiation between the fungal species. Figure 2.29 shows a PCA scores plot of the separation between the samples after 120 hours with the first two principal components accounting for approximately 94% of the variance in the data. Cluster analysis performed on the same data set using just the first two principal components also showed a similar result (Figure 2.30). The dendrogram was constructed as described previously. Although, four distinct groups can be visualised, one sample from C was clustered as belonging to R possibly an outlier.

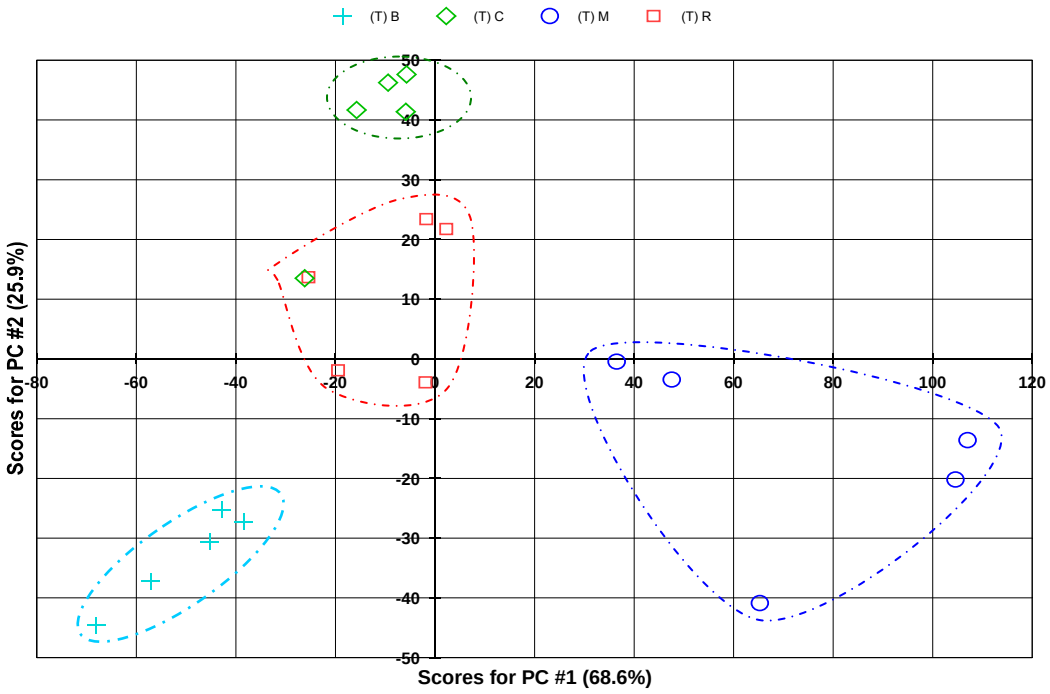



**Figure 2.29:** 120 hours PCA scores map indicating separation between the two human (M, R) and the animal (C) pathogens from the controls at 25°C on SDA.
(*Key:* M – *T. mentagrophytes*; R – *T. rubrum*; C – *M. canis*; B – controls)

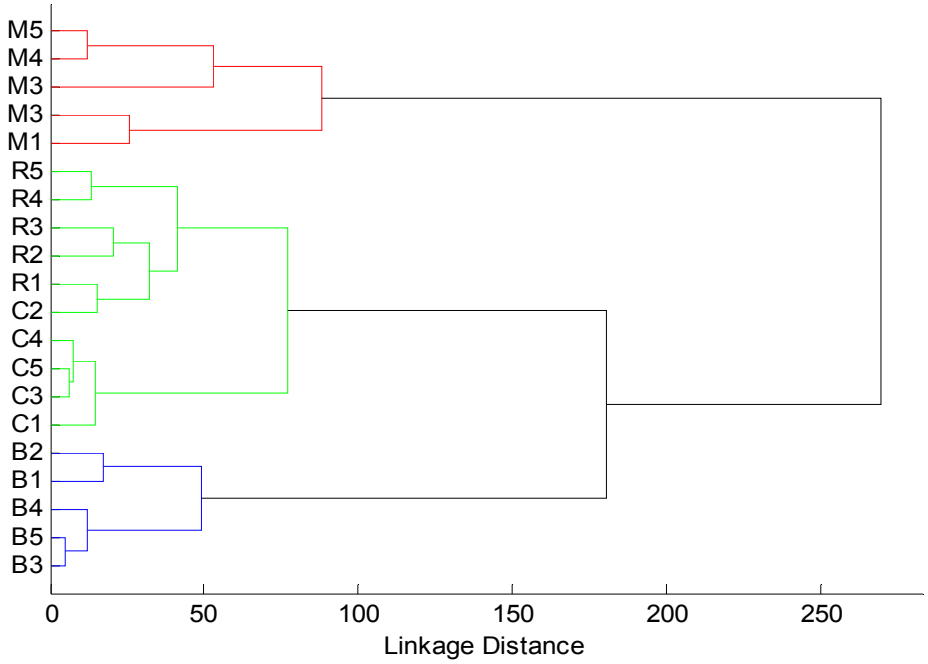



**Figure 2.30:** Tree diagram based on the first two PCs after 120 hours growth on SDA at 25°C showing four distinct clusters.
(*Key:* M – *T. mentagrophytes*; R – *T. rubrum*; C – *M. canis*; B – controls)

Additionally, just as described earlier, probabilistic neural networks were created in order to predict the classes of the fungal samples. The use of fewer sensors resulted in better classification accuracy, with spread values lower than 0.3. This can be seen in the confusion matrix in Table 2.3; which misclassifies one *M. canis* sample.

**Table 2.3:** Confusion matrix indicating 95% accuracy in predicting classes based on data using 18 sensors.
(*Key:* B – Controls; C – *M. canis*; M – *T. mentagrophytes*; R – *T. rubrum*)

| | | Predicted Class | | | |
|---|---|---|---|---|---|
| | | B | C | M | R |
| Actual Class | B | 5 | 0 | 0 | 0 |
| | C | 0 | 4 | 0 | 1 |
| | M | 0 | 0 | 5 | 0 |
| | R | 0 | 0 | 0 | 5 |

### 2.3.6 Mass spectrometric analysis

Analysis of the fungal samples (*T. mentagrophytes, T. rubrum* and *M. canis*) by GC-MS revealed the presence of numerous compounds in all the species including the agar controls. They comprised common volatiles such as alcohols (ethanol, butanol, 2-ethyl-1-hexanol), benzene derivatives (styrene, xylene, toluene) and 2-methyl-1,3-dioxolane to name a few including acetonitrile and propan-2-ol. However, it was observed that cyclohexane and 2-butanone were present in relatively higher amounts in the three fungi than in the controls; while acetone and methoxybenzene were much higher in *T. mentagrophytes* (although the latter was absent in agar and minutely present in the other fungi). On the other hand, 3-octanone and 1-octen-3-ol were found to be present in high concentrations in *T. rubrum* but only in trace amounts in the other two dermatophyte species (the former being possibly absent in *M. canis*) and absent in the controls. Examples of the chromatograms of each sample are shown in Figure 2.31 while their smaller peaks are magnified and illustrated in Figure 2.32.

Based on SIFT-MS analysis of the $H_3O^+$ precursor ion, one of the most striking observation was the production of huge amounts of ammonia, especially in *T. mentagrophytes* (≈ 6.3 ppm), followed by *T. rubrum* (≈ 1.15 ppm) and *M. canis* (≈ 0.6 ppm), although present in low levels (≈ 0.36 ppm) in agar. Volatiles such as dimethylamine and formaldehyde were detected in all fungal species at low ppb levels and possibly the presence of hydrogen cyanide in *T. rubrum*. As seen in GC-MS, certain common volatiles such as alcohols (methanol, propanol – low ppm levels and ethanol – low ppb levels) and traces of benzene derivatives were found in all samples. Acetone detected in *T. mentagrophytes* (≈ 3 ppm) was twice as much as the others, just as 2-butanone (based on $NO^+$ precursor ion) was found to be slightly higher in fungi (≈ 100

ppb) than in the agar (up to 45 ppb). Small amount of toluene was present in agar, but the fungi also had traces of toluene with more of propanoic acid present due to m/z 93 and 111. Certain unidentified compounds were present at m/z values of 70, 86, 90, 92, 98, 102, 114 and 126; although m/z 76 and 96 could be amines.

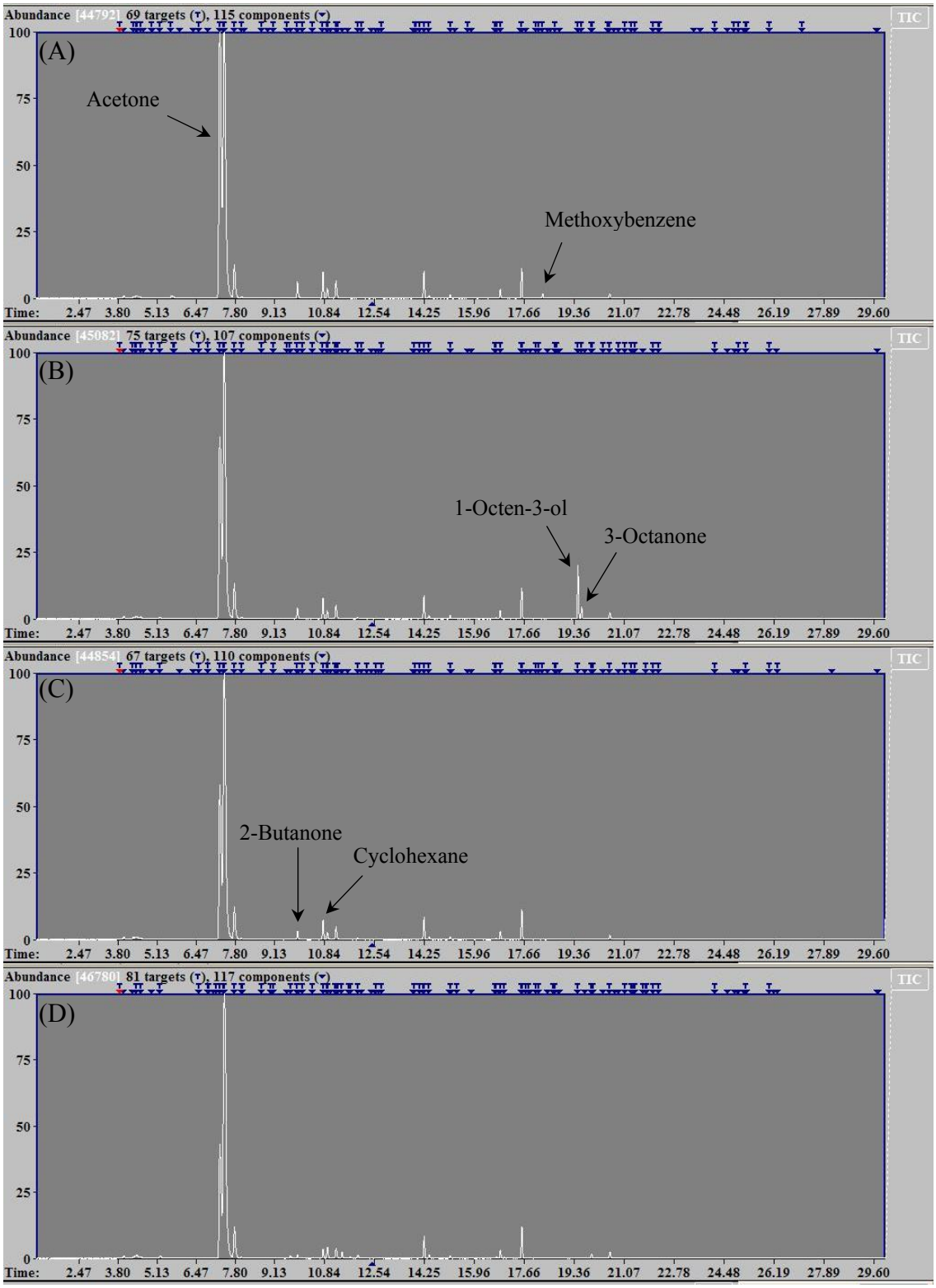



**Figure 2.31:** GC-MS chromatograms for *T. mentagrophytes* (A), *T. rubrum* (B), *M. canis* (C) and the agar control (D) after 96 hours incubation at 25°C.

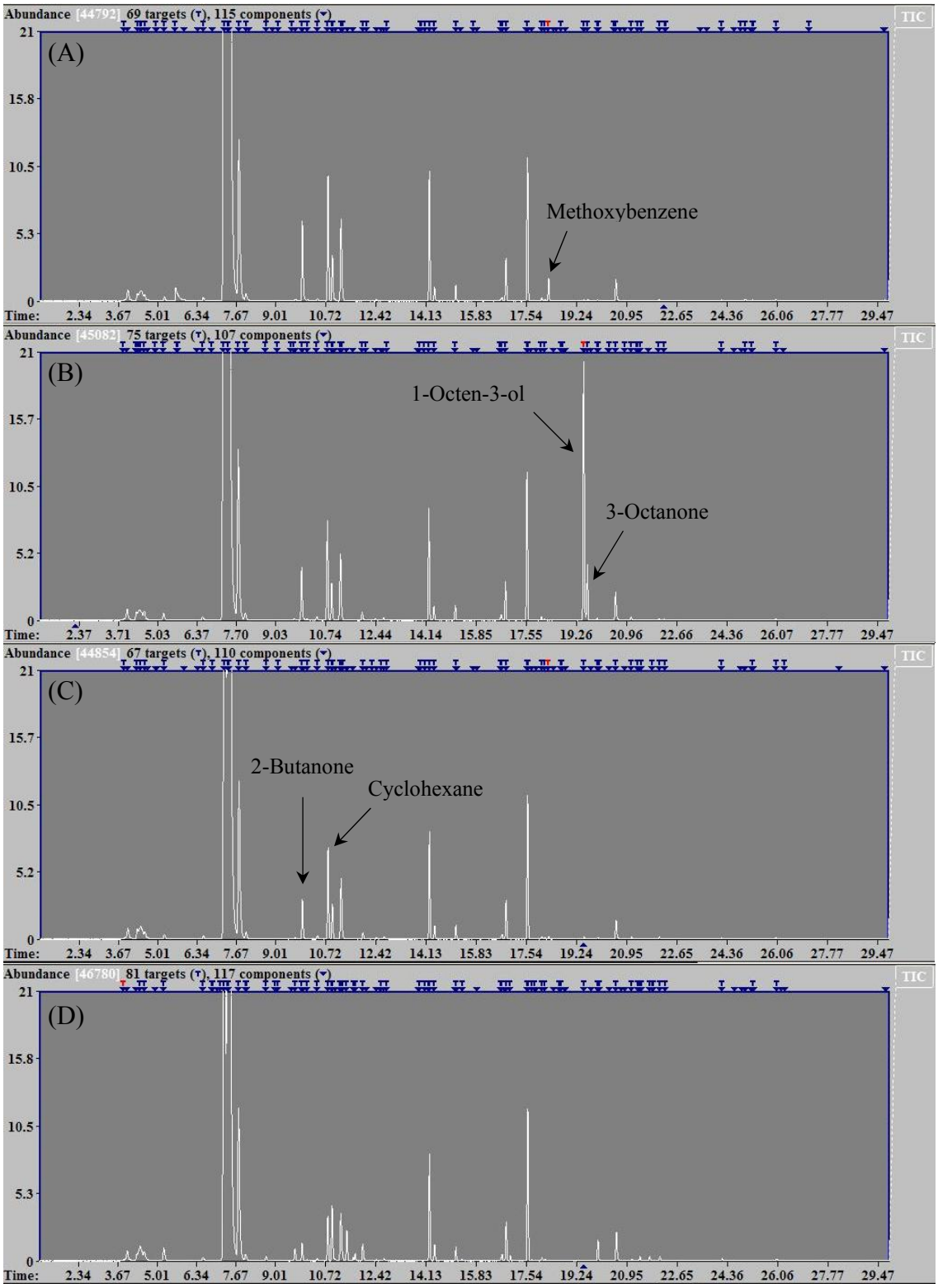



**Figure 2.32:** GC-MS chromatograms indicating the magnified smaller peaks from the Figure 2.31 for *T. mentagrophytes* (A), *T. rubrum* (B), *M. canis* (C) and agar (D).

## *2.4 Discussion*

This is the first study to examine volatile fingerprint profiles using electronic nose technology for discriminating between these medically important pathogenic fungi. It was observed that the hybrid sensor system outperformed the CP system in discerning the *Trichophyton* species, requiring about 72 hours to clearly distinguish two of the four species. However, in spite of using two different sampling procedures the CP system failed to segregate the pathogens from controls within 96 hours of incubation. This system may have performed better with a longer time period for volatile accumulation in the headspace, but, because the hybrid sensor system produced results earlier it was concluded that it would be more preferable for further studies. Moreover in clinical practice shorter time is ideal to decrease the total sampling time required thereby increasing efficiency.

Conducting polymers operate at ambient temperatures which make it conducive for a variety of applications, but are simultaneously very sensitive to humidity. This could have been a possible factor in this system's poor performance in the current study, but it is more likely that the headspace was not sufficiently concentrated. Nevertheless, previous studies were successful using this system (Gibson *et al.*, 1997; Pavlou *et al.*, 2002b; Canhoto & Magan, 2005).

Some studies carried out using the two types of sensors have also shown that there can either be no or minimal difference using these technologies or one proved to be better than the other. Nake *et al.* (2005) conducted experiments for outdoor air monitoring (*in situ*) of sewage odours by testing the two sensor technologies and found that the MOS sensors provided improved discrimination amid the various odours. On the other hand,

work done on monitoring the ripening of Danish blue cheese at an early stage using both MOS and CP sensors gave similar results (Trihaas & Nielsen, 2005; Trihaas *et al.*, 2005).

Successful intra- and inter-species discrimination, based on qualitative volatile profiles using agar media, between pathogenic dermatophyte species (*T. mentagrophytes, T. rubrum, T. verrucosum, T. violaceum* and *M. canis*) was shown to occur within 96-120 hours. Distinction of *T. mentagrophytes* and *T. rubrum* in broth was also possible after 72-96 hours using their volatile fingerprints.

Similar research, however, has been done on spoilage fungi in the food industry for early identification. Keshri *et al.* (1998) could differentiate grain spoilage moulds, i.e. *Eurotium, Penicillium* and *Wallemia* species within 48 hours by detecting their volatile odour patterns, however, reported that the two closely related *Eurotium* species could not be discriminated. Bread analogues spiked with various microbial species or lipoxygenase were subjected to electronic nose analysis where microbial spoilage could be distinguished from enzymatic spoilage after 48 hours (Needham *et al.*, 2005). These studies differentiated between the fungal species much earlier than traditional enzyme assays or plate count techniques (Keshri & Magan, 2000; Keshri *et al.*, 2002).

A recent study showed that volatiles from bacterial agar cultures were more effectively discriminated than broth cultures using an e-nose (Casalinuovo *et al.*, 2006). Magan *et al.* (2001), on the other hand, reported the detection and differentiation of bacteria and yeasts in milk-based media in about five hours. They could separate the various bacterial species and yeasts contaminating milk from each other as well as unspoiled

milk. Bacterial species distinction appears to take 24 hours or less as shown by Canhoto & Magan (2003) when detecting microbial populations and low concentrations of heavy metals in potable water.

Several studies discriminating the dermatophyte genera (*Epidermophyton, Microsporum,* and *Trichophyton*) have been previously carried out but at a genetic level using variations of PCR, PCR-RFLP and restriction enzyme techniques. Selection of random primers in the AP-PCR technique differentiated between the genera *Microsporum* and *Trichophyton* including selected species within these, except a few *Trichophyton* species. Although the authors found the two genera to be genetically similar, the DNA fragments produced were of different sizes facilitating distinction (Liu *et al.*, 1996; Liu *et al.*, 1997; Liu *et al.*, 2000). However, Liu *et al.* (2000) found that using a combination of primers increased the possibility of species identification except for *T. rubrum* and *T. gourvillii.* Differentiation of species has also been carried out using restriction enzymes by means of RFLP. Interestingly if the number of species studied were increased, certain discrepancies would probably arise especially if the restriction enzyme *HinfI* is considered (Shin *et al.*, 2003; Kamiya *et al.*, 2004). The former found that four *Trichophyton* species in two pairs could not be discerned from each other, while the latter distinguished between the three species used.

In another study, clinical samples were directly used for PCR-RFLP analysis without *in vitro* culturing where they were classified as either dermatophyte or *Scytalidium* species using primers specific for a restriction enzyme site in each species. The different dermatophyte species were not identified (Machouart-Dubach *et al.*, 2001). These studies suggested that very short time periods of 1-4 days for discrimination were

needed. Although a couple of recent studies have also directly used clinical samples using a shorter PCR method and reduced diagnostic time to either 48 or 5 hours (Kardjeva *et al.*, 2006; Brillowska-Dąbrowska *et al.*, 2007), these focus only on nail infections with a primary interest on *T. rubrum*. Also, most molecular techniques are carried out after culturing for a few days to 4 weeks. Moreover with the exception of diagnoses of a few common dermatophyte species, the other authors made use of various other species. The choice of primers also seemed to differ in each case which might not be feasible or cost effective on a wider clinical scale.

Additionally, the use of probabilistic neural networks in conjunction with volatile analysis resulted in good classification of the fungal species. Thus, it shows promise as a predictor of unknowns, although it might require further training due to the small sample size used in the current study. Larger sample numbers would enable better assessment of the network's generalisation capability. Studies have shown that RBF networks perform better and train faster than normal MLP networks. Panagou *et al.* (2007) predicted the growth rate of a spoilage fungus using an RBF network which showed that temperature was the most important factor. Other studies have also made use of such networks for taxonomic classification of a number of microalgae and phytoplankton especially using flow cytometric microbial features (Wilkins *et al.*, 1999; Al-Haddad *et al.*, 2000).

If species identification is important for administration of appropriate drugs, then strain differentiation can serve to be essential for monitoring drug resistance, especially in the event of treatment failure, in the strains of these species. Volatile profile patterns of the fungal strains of the two *Trichophyton* species indicate interesting outcomes. The

slower growth rate of some *T. rubrum* strains could explain the inability to discriminate it from the controls, suggesting that there is greater variability present in these species. This could also imply that the volatile patterns of the strains pertaining to these species might be slightly different from each other. In contrast, no distinction between the strains of *T. mentagrophytes* could be due to a great deal of similarity between the strains indicating that their volatile fingerprints hardly differ. These would however, need to be substantiated by MS techniques.

PCR techniques for dermatophyte strain typing in some instances have shown an inability to differentiate between *T. rubrum* strains (Liu *et al.*, 1996; Gräser *et al.*, 1999) or minor differences in a small number of strains (Zhong *et al.*, 1997). In contrast, other authors using primers from the nontranscribed spacer region of rDNA enabled strain differentiation of this species (Jackson *et al.*, 2000; Baeza *et al.*, 2006). However, Jackson *et al.* (2000) differ in opinion about the reproducibility of the technique used in the former study. Similar issues were reported in the case of *T. mentagrophytes* (Liu *et al.*, 1996; Kim *et al.*, 2000; Faggi *et al.*, 2001). A possibility for the varying reports could be the use of different primers in these studies.

Studies on fungal strain discrimination, especially those detrimental to the food industry due to toxin production, have been previously reported using different kinds of e-noses. Falasconi *et al.* (2005a) demonstrated that a MOS e-nose could classify the strains of *Fusarium verticillioides* based on their toxigenic behaviour on agar as well as on grain. Studies also showed that whilst black *Aspergilli* strains could be segregated, toxigenic *A. flavus* strains were not easy to differentiate based on volatile profiles (Cabañes *et al.*, 2006; Sahgal *et al.*, 2007). Furthermore, Needham and Magan (2003) used CP sensors

to examine *Penicillium verrucosum* strains for production of ochratoxin as opposed to those that did not produce the toxin.

Furthermore, detecting the threshold level of a fungal species can serve as an important indicator for early and rapid detection of pathogens. The minimum detectable limit for identifying either *T. mentagrophytes* or *T. rubrum* was found to be $10^3$ CFUs $ml^{-1}$ in broth as well as both solid culture media within 96 hours. Lower detection limits of $10^1$ CFUs $ml^{-1}$ can probably also be achieved at 120 hours, but there is a possibility that the sensors were saturated with the concentrated headspace from the higher inocula. This could also explain the increasing spread of these replicates after 96 hours. Preconcentration of the treatments might enhance the detection of lower thresholds.

Turin *et al.* (2000) developed a PCR assay for identifying pathogenic fungi which could detect minute amounts of fungal DNA from dermatological specimens, 10pg, corresponding to roughly 25 CFUs. However, the specimens included several species of yeasts and moulds apart from a mixture of species from the genera *Trichophyton* and *Microsporum.* Although sensitivity is high, species discrimination required integration of results from three different primer sets by superimposition/correlation. In comparison to the current study which deals with volatile compound measurements, it is very good. However, by improving and optimising current procedures by means of agitation or possibly spiking with enzymes or an alternative substance, low sample concentrations could be induced to produce more volatiles.

The minimum detection limit in various food matrices including milk as a medium was shown to be $10^3$-$10^4$ cells $ml^{-1}$ based on volatile organic compounds formed by micro-

organisms (Magan *et al.*, 2001; Magan & Sahgal, 2007). In contrast, microbial populations of bacteria and fungi of the order of $10^2$ CFUs were identified in potable water using qualitative volatile fingerprints (Canhoto & Magan, 2003; Canhoto & Magan, 2005). In these studies, it was shown that chemical contamination in conjunction with bacterial contamination changed the volatile profile using HS-SPME/GC analysis.

Volatiles detected by the MS techniques indicated the presence of specific compounds (e.g. methoxybenzene, 3-octanone, 1-octen-3-ol, ammonia) that could serve as potential identifiers of the fungi or perhaps the specific *Trichophyton* species; but none were detected that were solely present in a single dermatophyte. Moreover, the inability to detect low molecular weight compounds such as ammonia, formaldehyde and/or dimethylamine by GC-MS, was probably due to its poor sensitivity for substances within this range. In order to firmly establish the presence of biomarkers further work is needed, using a larger number of samples with the inclusion of strains if possible. Previously, Verscheure *et al.* (2002) used SPME-GC-MS for dermatophyte identification. However, these authors reported that only *M. gypseum* strains produced volatiles such as sesquiterpenes, butyrolactone, sulphur compounds and 1-octen-3-ol and/or 3-octanone (the final ones being identified in the present study mainly in *T. rubrum*); whilst others produced either butyrolactone only (some *Trichophyton* species) or no volatiles (*M. canis and M. cookei).*This is in contrast to the present study, where *M. canis* and the two *Trichophyton* species were shown to produce certain volatiles; however no sesquiterpenes were identified. Another study, attempted in using SIFT-MS for identifying certain medically relevant fungi, but also did not identify any specific markers, all the metabolites detected were common (Scotter *et al.*, 2005).

Recent research has shown that use of MS-electronic nose enabled detection of three fungal species in Spanish bakery products based on an ergosterol biomarker (Marín *et al.*, 2007). Karlshøj *et al.* (2007) differentiated between closely related mycotoxigenic fungi, especially *Penicillium*, and found that some species had similar volatile profiles using GC-MS mainly identifying ethanol, acetone, 2-methyl-1-propanol, 3-methyl-1-butanol and 2-pentanone.

This study has described a novel method using volatile fingerprinting for the rapid identification of dermatophytes. It shows potential for working with actual clinical samples from patients suffering from dermatophytosis especially at low fungal concentrations. The use of artificial neural networks would facilitate identification of unknown samples against the previously trained model. The successful application of this technique in clinical practice would dramatically reduce the otherwise time consuming conventional identification techniques and allow rapid administration of suitable drugs for treatment. Furthermore, the organic compounds produced by these species would definitely assist in developing a specific and portable e-nose that could be utilised with ease in a clinical setting.

# *Chapter 3*

# *ANTIFUNGAL SUSCEPTIBILITY USING VOLATILE FINGERPRINTS*

## *3.1 Introduction*

The most common skin diseases, that constitute public health concern worldwide, are caused by dermatophytes. They are mainly responsible for superficial and to some extent deep-seated infections of the keratinised tissue. Administration of appropriate antifungal therapy i.e. topical or systemic treatments is of critical importance and depends particularly on the species involved including the site and severity of infection. Besides these, the other contributing factors are the cost of drugs (oral therapy being more expensive), duration of treatment (longer for nails) and risks of increasing resistance in species (Hiruma & Yamaguchi, 2003; Gupta & Tu, 2006).

The existing methods adopted for detecting antifungal susceptibility against the dermatophytes are based on modifications of the reference methods for filamentous fungi or yeasts as specified by the NCCLS. This is due to the lack of a standardised protocol and has led to numerous investigations into developing suitable reproducible assays. Studies using broth microdilution tests have identified terbinafine to be the most potent commercial antifungal drug. However, these methods vary in terms of incubation periods and temperatures; but resulted in effectively similar minimum inhibitory concentrations (Fernandez-Torres *et al.*, 2002; Favre *et al.*, 2003; Santos *et al.*, 2006). Santos *et al.* (2006) also reported that inoculum preparation affected the antifungal susceptibility. Recently, Esteban *et al.* (2005) utilised and demonstrated the potential of a commercial system based on agar diffusion for antifungal susceptibility.

However, the risks associated with current commercial therapeutics - such as drug-drug interactions, unpleasant side-effects and escalating fungal resistance – have led certain researchers to focus on naturally occurring sources e.g. various plant products for

antifungal activity, although the techniques used were similar to those mentioned previously (Gurgel *et al.*, 2005; Koc *et al.*, 2005). Studies have also shown essential oils to have inhibitory effects on dermatophyte growth including significantly boosting the effects of an antifungal when used in combination (exhibiting synergism) (Shin & Lim, 2004; Pyun & Shin, 2006).

These procedures tend to be tedious because of the difficulty in determining the end point for the inhibitory concentrations. Therefore, the present study determined the potential of a sensor array system to screen antifungal agents at different conditions (such as concentrations and temperatures) based on their generated volatile fingerprints.

## *3.2 Materials and methods*

### 3.2.1 Strains and antifungal agent

The fungal strains selected for this study were type cultures of two important *Trichophyton* species viz, *T. mentagrophytes* (NCPF-224) and *T. rubrum* (strain D12). Itraconazole (ITZ), the antifungal agent selected for the screening study was kindly provided by Janssen Pharmaceutica, Belgium.

The cultures were grown and maintained on Sabouraud Dextrose Agar (SDA) prepared in house by mixing 10 g $l^{-1}$ Mycological peptone (Amersham), 40 g $l^{-1}$ Glucose (Acros Chemicals) and 15 g $l^{-1}$ Agar technical no. 3 (Oxoid). 0.05 g $l^{-1}$ of the antibiotic, Chloramphenicol (Sigma) was also added. The antifungal stock solution was prepared in dimethyl sulfoxide (DMSO) (Sigma).

### 3.2.2 Temporal growth study

The efficacy of the antifungal was tested by measurements of the effect on temporal growth. The antifungal was screened at 0.25, 0.5, 1 and 2 ppm on SDA at 25 and 30°C. The treatments were prepared from 100 ppm stock solution in DMSO by adding to molten media to obtain the final concentrations. Controls were fungi inoculated on plain SDA media and uninoculated SDA media. The plates were centrally inoculated with a 4mm agar plug from three week old cultures of both *T. mentagrophytes* and *T. rubrum*. Three replicates per treatment were incubated at 25 and 30°C. Colony diameter was measured in two directions at right angles to each other for up to two weeks.

### 3.2.3 E-nose volatile fingerprint analysis

Spore suspensions of three to four week old actively growing cultures were prepared in sterile 10 ml Tween 80 (Acros Chemicals) and RO water. The initial inoculum concentrations measured using a haemocytometer microscopically were in the range of $10^7$ spores $ml^{-1}$ for *T. mentagrophytes* and *T. rubrum* respectively. Two sets of experiments were performed. In the first set, five replicate agar plates for each treatment ($LD_{50}$[‡‡] and controls) per species were inoculated with 250 µl of the inoculum. They were incubated at 25 and 30°C for 96 hours following which the five replicates were destructively sampled. Blank agar plates including some containing the antifungal were used as negative controls, to ensure that it had no effect on the volatile profiles that would be responsible for any kind of discrimination. For the second experiment, the treatments used were the $LD_{50}$ values, 2 ppm (approx. 90% inhibition i.e. $LD_{90}$) and controls per species, incubated only at 25°C and analysed as described earlier except that they were sampled after 96 and 120 hours.

[‡‡] $LD_{50}$ – the effective concentration of the antifungal at which the fungal growth is 50% inhibited.

Four 2 cm diameter agar plugs were placed in 25 ml vials and set aside to equilibrate for an hour at 25 and 30°C for headspace generation. The sample vials were placed in the hybrid e-nose (NST 3320, Applied Sensor, Sweden) carousel system and analysed in a random order. The headspace of the samples was measured by exposure to the sensor array after uptake by a robotic needle (details in Appendix B, B.1). The baseline for the system was set by passing air through the activated carbon filter. After sample uptake the system was flushed with air again to prevent carry over effects. These studies were repeated at least twice.

### 3.2.4 Data analysis

Fungal growth rate measurements against the antifungal were used to determine the lethal dose at which the fungi were 50% inhibited ($LD_{50}$ value) using Microsoft® Excel. The subsequent e-nose sensor data (mean-centred) were analysed with the accompanying software NSTSenstool and Statistica 7 (Statsoft Inc.) using multivariate statistics such as principal component analysis (PCA) and cluster analysis (CA) respectively. In addition, PCA loadings plots were also examined for selection of suitable sensors. The patterns in the data, if any, are depicted in the form of scores plots and tree diagrams i.e. dendrograms.

## *3.3 Results*

### 3.3.1 Temporal effects of antifungal screening

Temperature and concentration of the antifungal agent affected the growth rates of the two fungal species. The fungal controls appeared to grow slightly faster at 30 than at 25°C. At 25°C the growth of *T. mentagrophytes* and *T. rubrum* was almost completely inhibited by the antifungal concentration of 2 ppm, i.e. $LD_{90}$ (Figure 3.1). At the higher

temperature however, the trend for inhibiting the growth of the fungi at the intermediate concentrations was different (Figure 3.2). The effective concentrations at which 50% of fungal growth was inhibited was determined from these data and shown in Table 3.1.

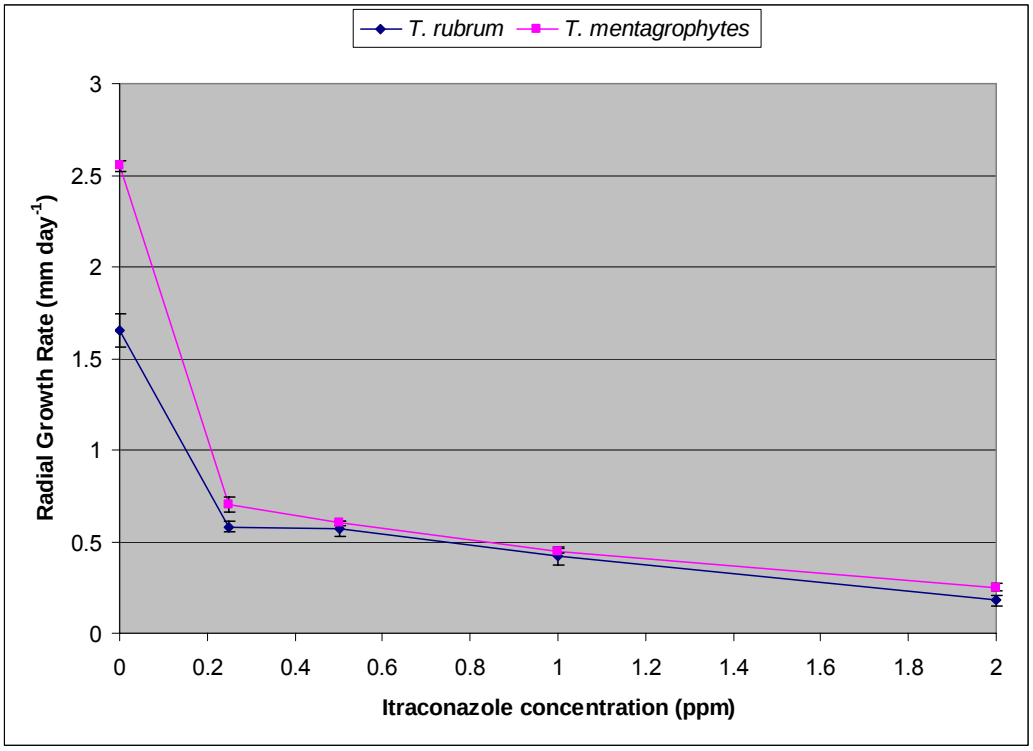



**Figure 3.1:** *In vitro* effect of the varying antifungal (itraconazole) concentrations at 25°C on the growth rate of the two dermatophyte species (I – standard error bars).

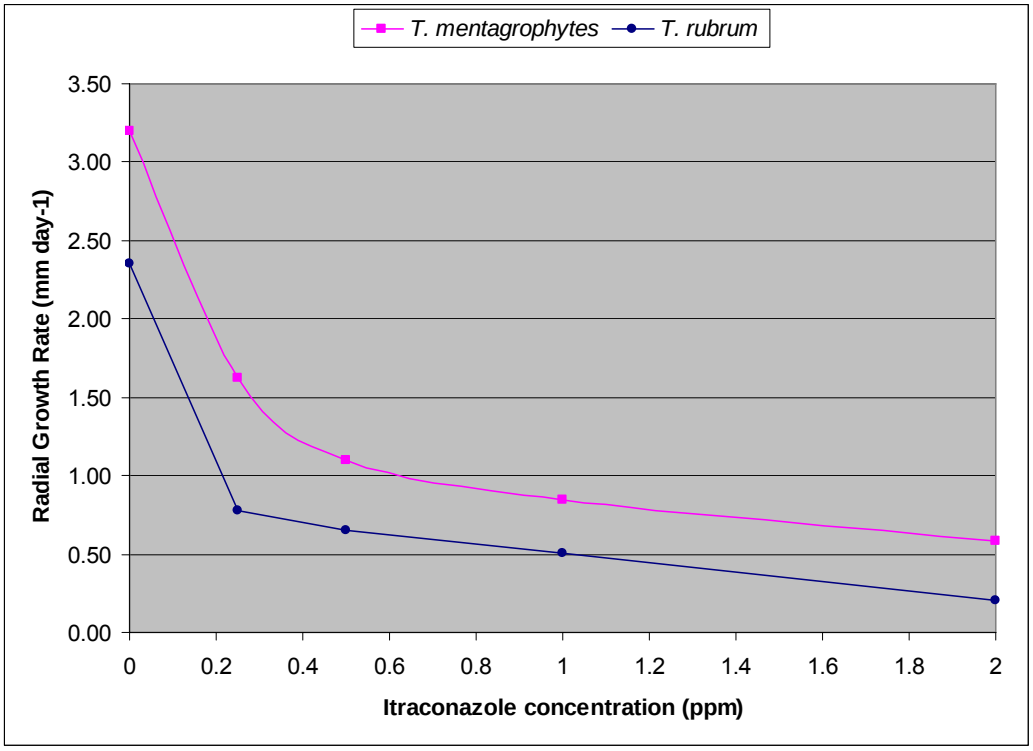



**Figure 3.2:** Radial extension rates of the two fungi, *T. mentagrophytes* and *T. rubrum* based on the effect of different antifungal concentrations at 30°C.

**Table 3.1:** Antifungal concentrations at which 50% (mycelial) growth is inhibited at different temperatures relative to the untreated controls.

| Temperature | ITZ $LD_{50}$ concentration (ppm) | |
|---|---|---|
| | *T. mentagrophytes* | *T. rubrum* |
| 25 °C | 0.16 | 0.18 |
| 30 °C | 0.22 | 0.14 |

### 3.3.2 Antifungal volatile profiles

Based on the initial screen at 25°C, an antifungal concentration of 0.16 ppm and 0.18 ppm was used for the e-nose study of the two dermatophytic fungi respectively. Figure 3.3 illustrates the PCA scores plot where the two species without antifungal can be

differentiated from each other and the controls; but the antifungal treatments are grouped together after 96 hours analysis. The first two PCs accounted for approximately 98.8% variance in the data. However, cluster analysis (using Euclidean distance and Ward's linkage) on the data showed two main clusters segregating antifungal treatments from non-antifungal treatments. In case of the latter, only the *T. mentagrophytes* samples formed a distinct cluster that differentiated them from the controls, but samples belonging to the controls and *T. rubrum* were not (Figure 3.4).

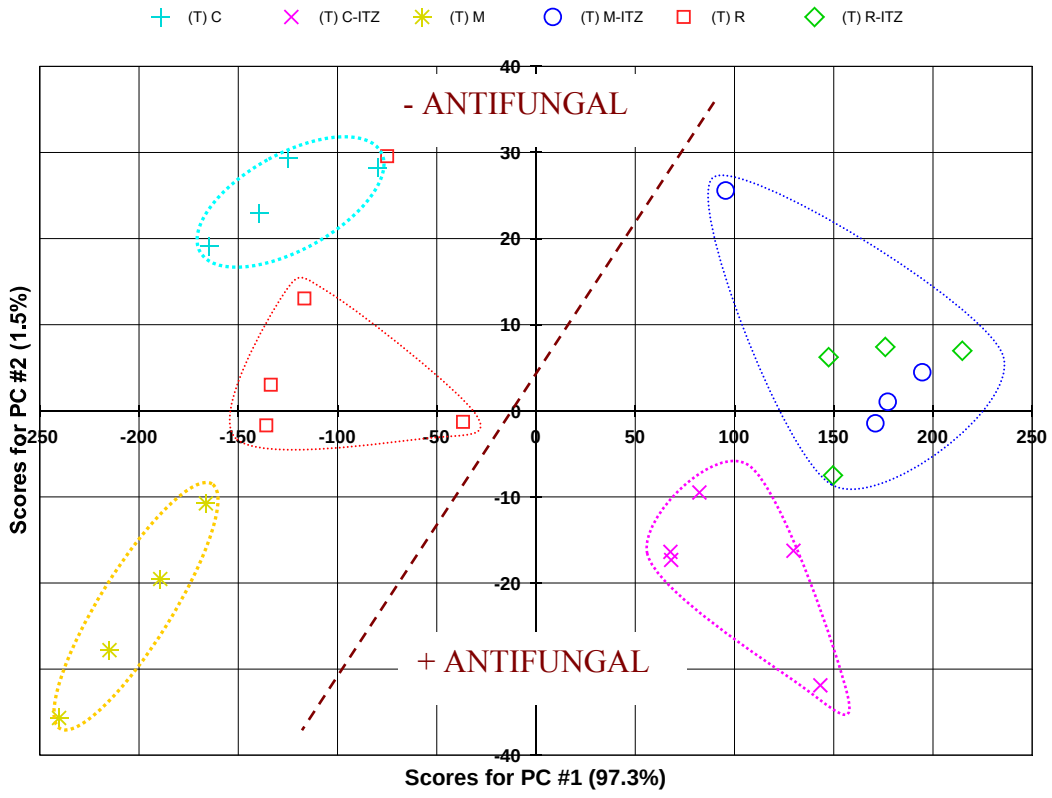



**Figure 3.3:** PCA plot depicting the effect of the presence of antifungal (itraconazole, ITZ) on sensor responses of the two fungal species after 96 hours incubation at 25°C. (*Key:* C – Controls; C-ITZ – Controls + antifungal; M – *T. mentagrophytes*; M-ITZ – *T. mentagrophytes* + antifungal; R – *T. rubrum*; R-ITZ – *T. rubrum* + antifungal)

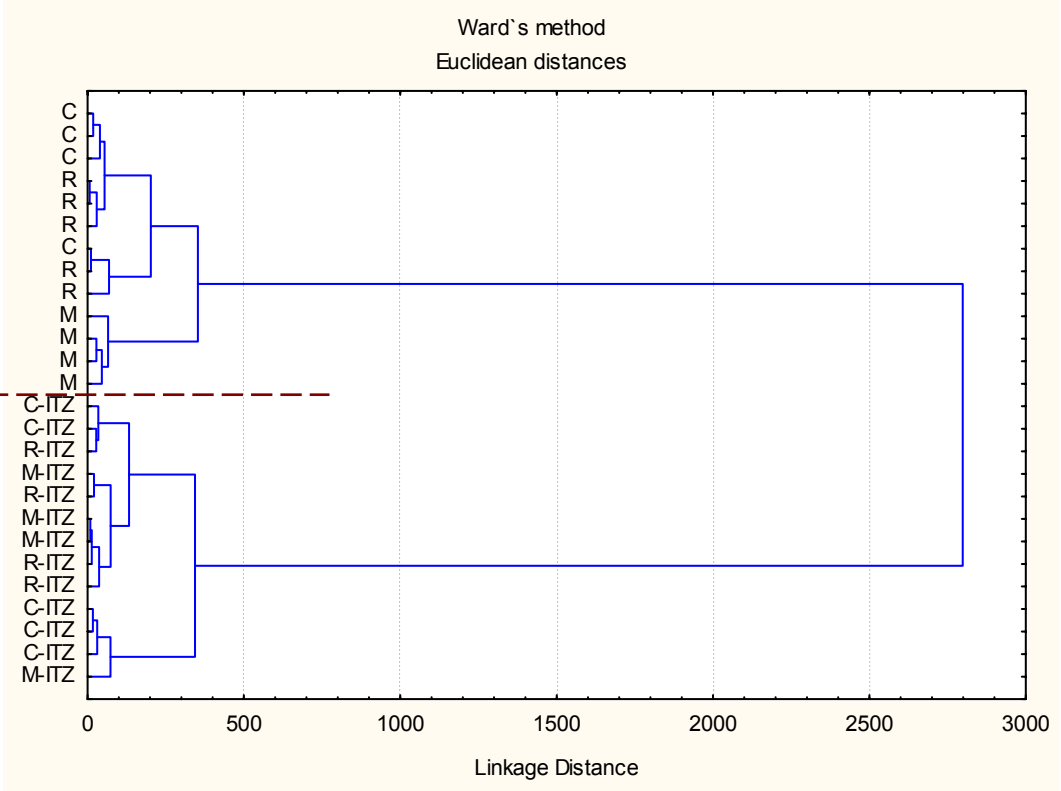



**Figure 3.4:** Dendrogram depicting two distinct clusters separating antifungal treatments from those without antifungals including only *T. mentagrophytes* (without antifungal) being clearly differentiated (at 25°C).
(*Key:* C – Controls; C-ITZ – Controls + antifungal; M – *T. mentagrophytes*; M-ITZ – *T. mentagrophytes* + antifungal; R – *T. rubrum*; R-ITZ – *T. rubrum* + antifungal)

The antifungal concentrations used for similar experiments at 30°C for the two fungal species were 0.22 ppm and 0.14 ppm respectively. Analysis of the sensor responses after 96 hours growth based on PC1 and PC2 indicated that the fungi, especially *T. rubrum*, in the absence of the antifungal could be differentiated from each other and the controls. The other treatments with ITZ were dispersed among the controls and one fungal species (Figure 3.5A). On the other hand, when observing the first and fourth PCs except for the *T. mentagrophytes* samples treated with the antifungal that were scattered, the remaining treatments appeared to be differentiated from each other (Figure 3.5B). In either case, over 90% of the variance in the data was accounted for.

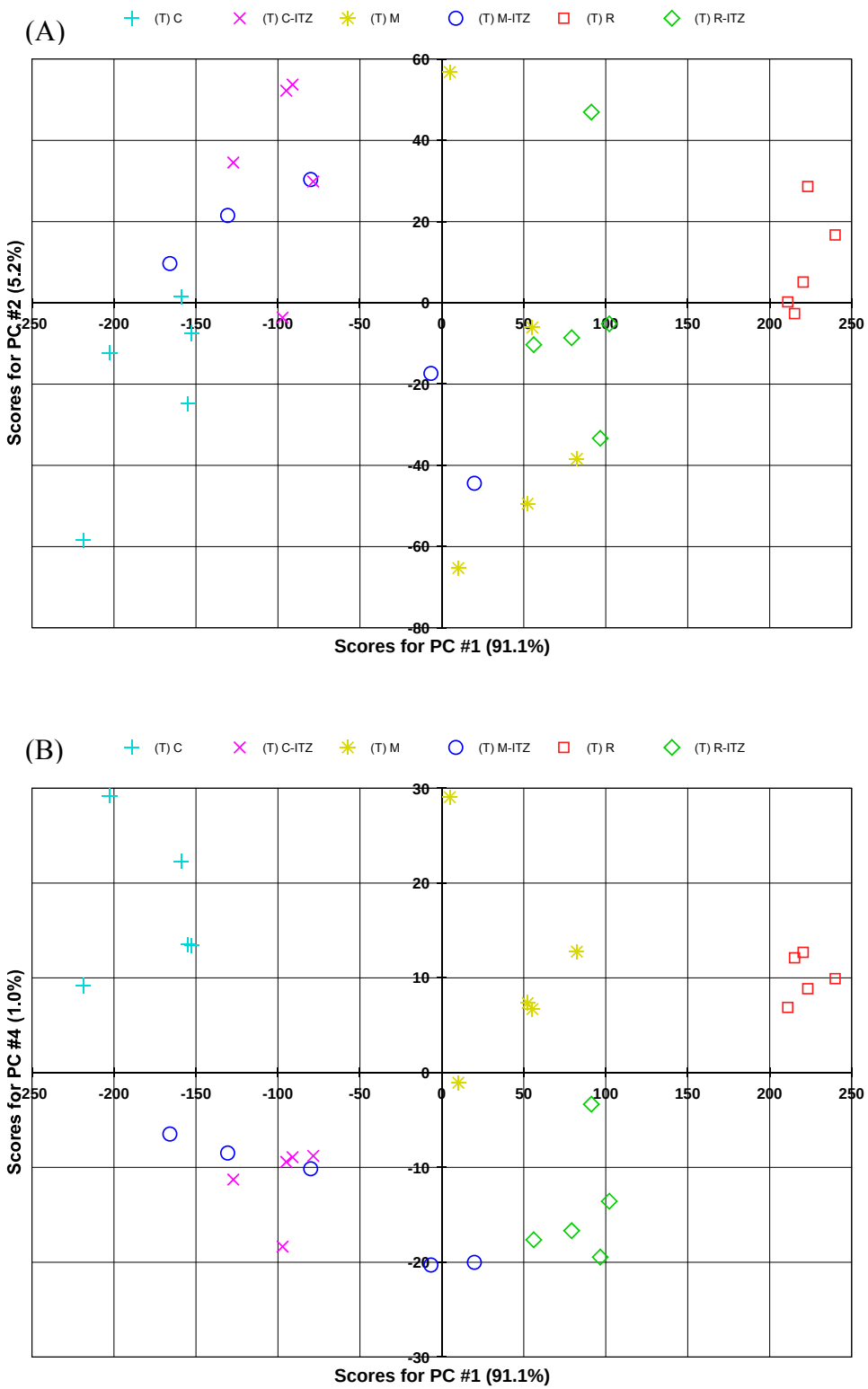



**Figure 3.5:** PCA analysis of fungal samples treated with and without an antifungal at 30°C after 96 hours observing PC1 vs. PC2 (A) but PC1 vs. PC4 (B) indicate better differentiation between treatments.

(*Key:* C – Controls; C-ITZ – Controls + antifungal; M – *T. mentagrophytes*; M-ITZ – *T. mentagrophytes* + antifungal; R – *T. rubrum*; R-ITZ – *T. rubrum* + antifungal)

Subsequent analysis of sensor responses on data from two itraconazole treatments - $LD_{50}$ and 2 ppm, i.e. $LD_{90}$ and controls after 96 hours fungal growth at 25°C showed that the fungal treatments exclusive of itraconazole could be discriminated from the remaining treatments (Figure 3.6). After 120 hours *T. rubrum* and *T. mentagrophytes* without the antifungal agent could be differentiated from each other much clearly and the remaining treatments. Nevertheless, the controls and treatments with both the concentrations of the antifungal agent could not be segregated even when three PCs were considered (Figure 3.7). The three PCs accounted for approximately 97% of the variance in the dataset. Cluster analysis on the data is shown in Figure 3.8 which substantiated the above.

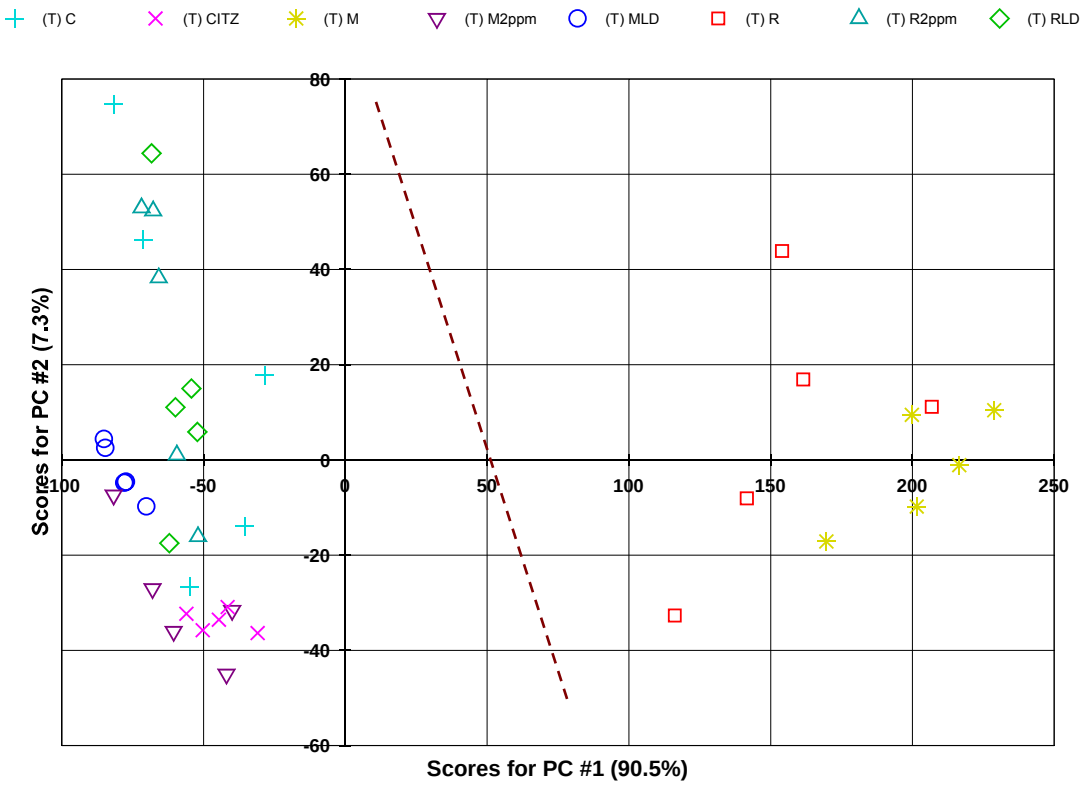



**Figure 3.6:** PCA plot showing discrimination between fungal treatments without the presence of itraconazole from those with, including the controls after 96 hours at 25°C. (*Key:* C – Controls; CITZ – Controls with 2ppm antifungal; M – *T. mentagrophytes*; R – *T. rubrum*; M/R-LD – Fungi with $LD_{50}$ concentration; M/R-2ppm – Fungi with 2 ppm, i.e. LD90, antifungal concentration)

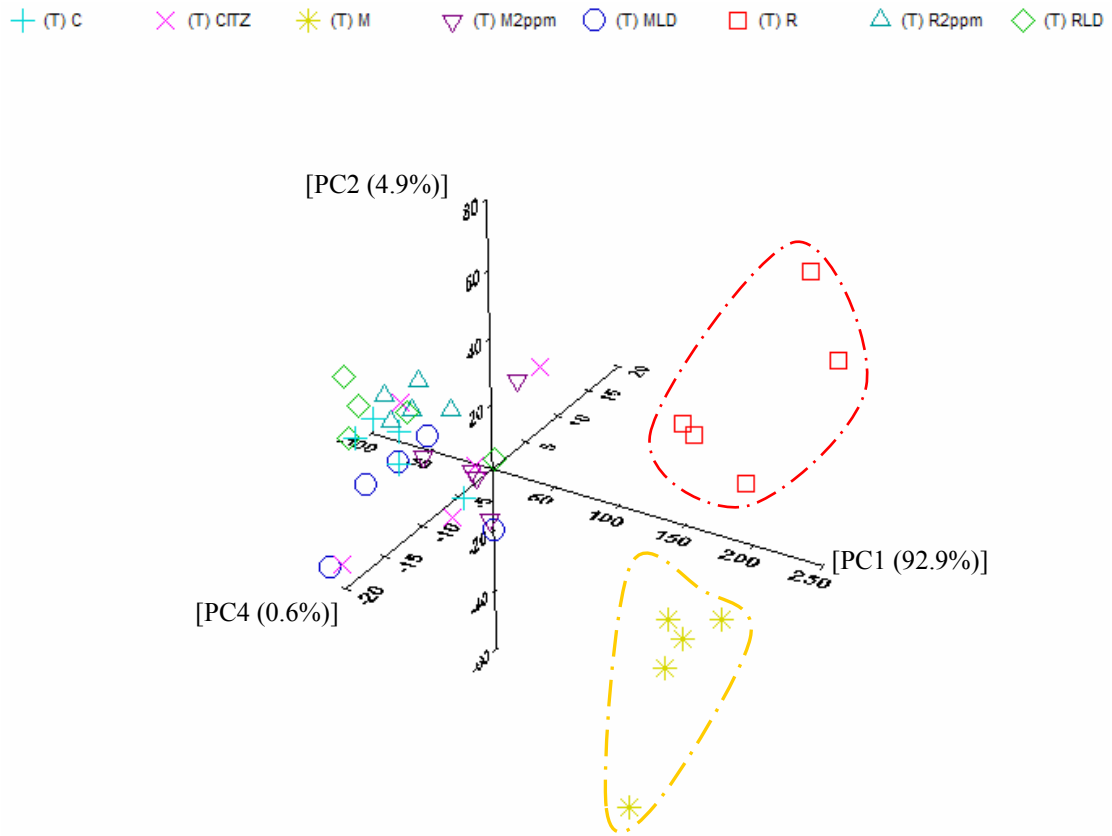



**Figure 3.7:** 3D PCA plot illustrating discrimination between the two fungal species, after 120 hours at 25°C, in the absence of itraconazole from each other and the remaining treatments including controls.

(*Key:* C – Controls; CITZ – Controls with 2ppm antifungal; M – *T. mentagrophytes*; R – *T. rubrum*; M/R-LD – Fungi with $LD_{50}$ concentration; M/R-2ppm – Fungi with 2 ppm, i.e. $LD_{90}$, antifungal concentration)

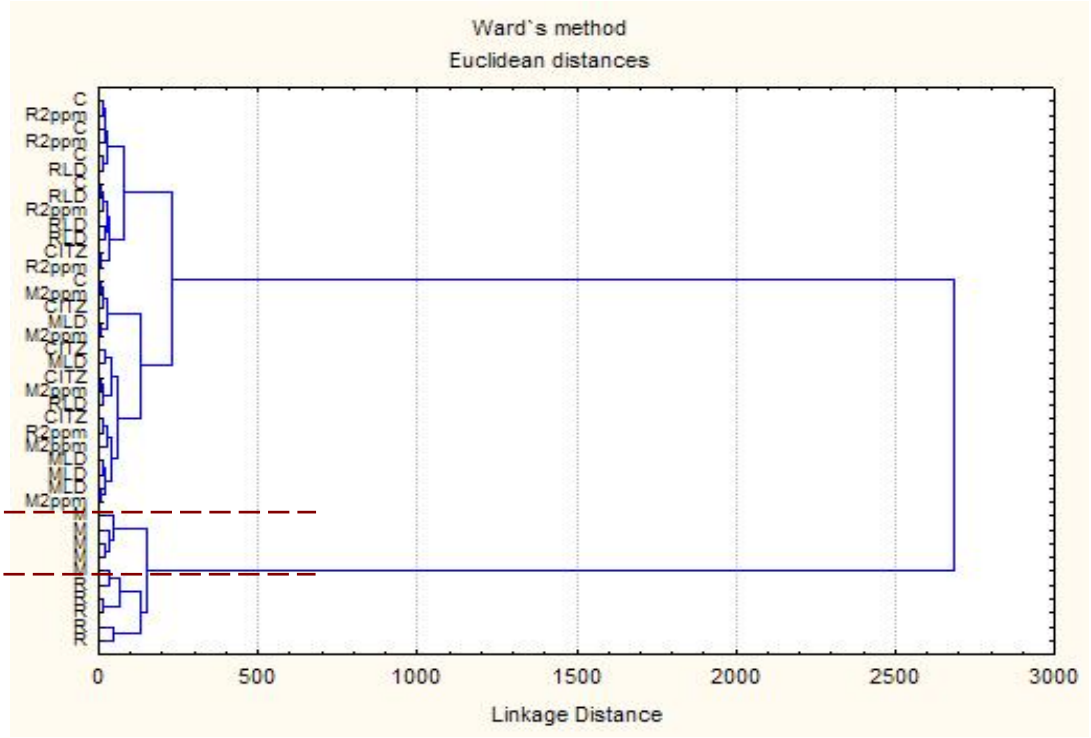



**Figure 3.8:** Dendrogram, after 120 hours fungal growth at 25°C, showing clear discrimination between the two dermatophytes in the absence of itraconazole from the other treatments with the antifungal agent.

(*Key:* C – Controls; CITZ – Controls with 2ppm antifungal; M – *T. mentagrophytes*; R – *T. rubrum*; M/R-LD – Fungi with $LD_{50}$ concentration; M/R-2ppm – Fungi with 2 ppm, i.e. $LD_{90}$, antifungal concentration)

## *3.4 Discussion*

The efficacy of the antifungal agent, itraconazole was determined *in vitro* on the two main dermatophyte species at two different temperatures. At the higher temperature, the fungi appeared to grow relatively faster, however for achieving 90% inhibition of growth *T. mentagrophytes* required > 2 ppm of the chemical at 25°C. For *T. rubrum* the same level of inhibition was achieved at both temperatures with 2 ppm of the treatment. Furthermore, there was not much change in the rate of growth of both fungi at 25°C between 0.25 and 0.5 ppm of the antifungal. In contrast, previous studies have reported

a stimulation effect of antioxidants and essential oils at intermediate concentrations on growth and toxin production of certain fungi *in vitro* and on grain (Hope *et al.*, 2002). This effect was observed at 15 and 25°C. As these studies were on cereal grain, the effects at higher temperatures were not relevant. In addition, the effect of an antifungal treatment which gave 50% inhibition of growth was different for the dermatophytes at the two temperatures i.e. *T. mentagrophytes* required a higher concentration of itraconazole at 30 (0.22 ppm) than at 25°C (0.16 ppm) and vice versa in the case of *T. rubrum*.

The initial screening process was based on temporal studies, thereafter the susceptibility of the fungi to itraconazole was determined by their *in vitro* volatile profile patterns with and without the antifungal agent. E-nose analyses after 96 hours growth at either temperature differentiated between treatments with the antifungal and those without the agent. However, at 25°C the discrimination was more prominent than at 30°C because at the higher temperature there was a greater spread in some of the treatments (for e.g. *T. mentagrophytes* plus antifungal). Furthermore, there appeared to be contrasting observations for the fungal control samples at the two temperatures. *T. mentagrophytes* formed a distinct cluster at the lower temperature whilst in case of the higher temperature it was *T. rubrum*.

A difference in the clustering pattern of the fungal treatments with itraconazole was also noticed, with there being no distinction between them at 25°C; but present at 30°C for the set of *T. rubrum* samples especially when other PCs were considered. This showed the importance of analysing the effects of other PCs on the samples that could be due to the subtle differences in loadings (variables). Those belonging to other fungal species

were however still scattered. There also appeared to be some effect on the sensors in the presence of itraconazole, as segregation to some extent could be seen between the negative controls i.e. uninoculated media with and without the addition of the antifungal.

The fact that there was no discrimination between the controls and treatments containing two antifungal concentrations could be explained by virtually no growth or inhibition of the fungi at 2 ppm of the antifungal (after 96-120 hours), thus being very similar to the uninoculated controls. The inability to distinguish the second antifungal concentration (responsible for 50% inhibition) which was still similar to the controls as in the other experiments probably implies that longer incubation times are required. Previous studies by Fernandez-Torres *et al.* (2002) have indicated an optimum incubation period of seven days before visual inspection when traditionally screening antifungals against dermatophytes. However, at 120 hours, both the fungal controls were clearly distinguished from the negative controls and treatments with antifungals.

Needham (2004) used an e-nose to screen the efficacy of antioxidants and preservatives such as butylated hydroxyanisole and propyl paraben *in vitro* for their use in the food industry. Her study indicated the ability of the e-nose to discriminate between treatments containing antioxidants, responsible for at least 50-70% inhibition in growth, from those without when individually inoculated with different micro-organisms. Antifungal screening for dermatophytes, to date has been performed using variations of the broth microdilution technique as specified by the NCCLS. However, these studies made use of different dermatophyte strains and modified the methodology including a varying range of temperatures (28 to 30°C) and incubation periods (3 to 7 days)

(Fernandez-Torres *et al.*, 2002; Favre *et al.*, 2003; Santos *et al.*, 2006). Santos *et al.* (2006) reported that filtering the inoculum suspension before use resulted in homogeneous fungal growth and lowered the minimum inhibitory concentration of the current antifungal drugs. Thus, suggesting that fungal hyphae are not as susceptible as microconidia to the antifungal treatments.

Similar adaptations were carried out for screening plant extracts and essential oils as antifungals against dermatophytes using the traditional tests. These fungi have been reported to display varying susceptibility to plant extracts; especially *T. violaceum* (Ali-Shtayeh & Abu Ghdeib, 1999) and *T. rubrum* (Silva *et al.*, 2005), although most of the fungal species and medium used differed in the two studies. Reports have also indicated different plant extracts to have a lower or similar efficacy when compared with commercial antifungals such as griseofulvin (Ali-Shtayeh & Abu Ghdeib, 1999; Gurgel *et al.*, 2005). However, Silva *et al.* (2005) showed eugenol extract from *Ocimum gratissimum* (wild basil) to have a higher antifungal activity than itraconazole at the same concentration because the former inhibited the growth of other dermatophytic isolates.

This is the first study that has assessed the potential of volatile fingerprints to screen for antifungal susceptibility with an e-nose. The e-nose had the ability to distinguish between fungal controls without itraconazole from those containing the compound. Nevertheless, in order to use it as a method for screening antifungals, it needs to be further explored so that at least a certain level of growth inhibition can be discriminated. Furthermore it could also serve as a useful tool for checking resistance build up against the antifungal amongst the different species and/or strains of the dermatophytes.

# *Chapter 4*

# *EARLY DIAGNOSIS OF VENTILATOR ASSOCIATED PNEUMONIA*

## *4.1 Introduction*

Identifying infections or diseases based on the 'smell' generated is fast gaining popularity as a non-invasive clinical diagnostic procedure. It is well known that certain diseases produce metabolic products that have characteristic odours, ranging from acetone breath in diabetics or fishy smell for liver disease to bacterial infections with distinctive odours. However, not all of such odours are perceptible by the human nose especially at the initial stages, resulting in traditional microbiological diagnosis – which is laborious, time-consuming and requires a high degree of skill. Therefore, over the recent years research has exploited devices such as electronic noses as an alternative for rapid identification of bacterial infections.

Recent studies on tuberculosis have successfully demonstrated the use of an e-nose for discriminating between various Mycobacterial isolates in humans (Pavlou *et al.*, 2004) and in animals (Fend *et al.*, 2005). Dutta *et al.* (2005) showed that three groups of staphylococci responsible for ear-nose-throat (ENT) infections could be identified using an e-nose in combination with neural networks. Similar *in vitro* studies have also been carried out on bacteria causing eye infections (Boilot *et al.*, 2002; Dutta *et al.*, 2002).

One of the most common and severe hospital acquired infections is ventilator associated pneumonia (VAP). It has a high rate of mortality and has increased incidence in critically ill individuals. Due to the absence of a gold standard technique, VAP is difficult to accurately diagnose. The present diagnostic methods comprise of microbiology and mainly bronchoscopic or endotracheal methods which are either not very accurate or invasive. Furthermore, failure in precise diagnosis leads to

inappropriate antibiotic therapy which in turn has the risk of increasing drug resistant pathogens.

This study explored the potential of volatile fingerprints generated by the various micro-organisms not only *in vitro* but also those present within the clinical samples using an e-nose in discriminating between these species. It also attempted to correlate the microbiology culture results with the e-nose responses for the clinical samples.

## *4.2 Materials and Methods*

### 4.2.1 Collection of clinical samples

Patients recruited for this study were mechanically ventilated for more than 72 hours, at the Gloucestershire Royal Hospitals. The control group comprised those that were ventilated for less than 24 hours for any reason. The patient groups underwent a blind bronchoalveolar lavage (BAL) performed by the clinician on day three for the experimental group and within the first day for the control group (ethics approval in Appendix D, D.1).

The procedure involved introducing 20 ml sterile normal saline into the lungs by means of a sterile catheter attached with a 20 ml syringe. The solution was injected and immediately aspirated back. In order to avoid contamination from microbes colonising the tubes, precaution was taken not to aspirate during catheter withdrawal. The aspirate was collected in sterile containers and then divided, with one set being sent for standard microbiological analysis (hospital labs) and the second set (up to 5 ml) posted to the University for volatile analysis (blind study). The samples were stored at 4°C prior to

analysis. A total of 67 samples were collected over a period of 13 months (May 2006 to June 2007).

### 4.2.2 Microbial isolates

A total of 16 microbial species (such as *Staphylococcus*, *Streptococcus*, MRSA, *Proteus*, *Pseudomonas*, *Klebsiella*, *Enterobacter* and *Candida*) isolated from mechanically ventilated patients in the intensive care units at the Gloucestershire Royal Hospitals were obtained from the Cheltenham General Hospital (specific details can be found in Appendix A, A.2). These were maintained on nutrient agar (LabM) at 37°C for this study and stored at 4°C until required.

### 4.2.3 Headspace analysis

The clinical samples were removed from the cold room and allowed to thaw at room temperature. Following which the samples were pipetted into 25 ml glass vials and set aside for an hour to equilibrate at room temperature. The headspace was then analysed in a random order using an e-nose, the NST 3320 (Applied Sensor, Sweden) – comprising a hybrid sensor array.

For the microbial species, the isolates were maintained on nutrient agar plates. One colony per species was initially inoculated in 10 ml sterile nutrient broth (LabM) and incubated for four hours at 37°C in a rotary shaker at 100 rpm. Thereafter 100 µl of each microbial suspension was transferred into fresh 10 ml sterile nutrient broth and incubated for 18 hours at 37°C in a rotary shaker at 100 rpm. Subsequently, 5 ml from each suspension (approximately $10^8$ and $10^6$ cfu $ml^{-1}$ for the bacterial and yeast species respectively) was transferred into 25 ml glass vials, set aside for one hour at 37°C for

headspace generation. Uninoculated nutrient broth was used as controls and five replicates per treatment were analysed in a random order using the e-nose. These studies were repeated at least twice.

### 4.2.4 Data analysis

The responses generated by the e-nose sensors, in the form of normalised, mean-centred data for the response parameter, were analysed by Matlab 7.2 (Mathworks Inc.). Multivariate statistics involving principal component analysis (PCA) and linear discriminant analysis (LDA) were applied to the data in order to identify any possible relationships between sample treatments and check the prediction capability. The results were displayed in the form of scores plots.

In case of the clinical samples, the PCA scores plots were analysed and the results were then correlated to the findings obtained from microbial analysis on the patient's lavage samples. This was done with the help of the clinician involved in the entire study based at the Gloucester Royal NHS Hospital. At no point were the patients' identity disclosed, all analysis and correlations were based on identification numbers.

## *4.3 Results*

### 4.3.1 Discrimination of the microbial isolates

For the *in vitro* studies the bacterial and yeast species could be discriminated from the controls, but not all of the microbial species could be distinguished from each other (Figure 4.1). Within this thirteen group system it was observed that mainly the *Klebsiella*, *Enterobacter* and *Proteus* species could be clearly differentiated, whilst the remaining groups were rather tightly clustered. This was however further justified on

considering a four group system viz. gram positive, gram negative, fungi and controls. Figure 4.2 depicts a PCA scores plot of these four groups, which indicated that the gram negative organisms were best differentiated from the others. The three PCs accounted for approximately 90% of the variance in the data in both instances. Upon supervised analysis using LDA, a relatively clearer segregation was observed based on the four group model as seen in Figure 4.3. It was seen that 81 of 98 samples were correctly classified (i.e. a classification accuracy of about 83%). The classification matrix and the distance measures between the four groups based on the supervised technique are shown in Tables 4.1 and 4.2 respectively.

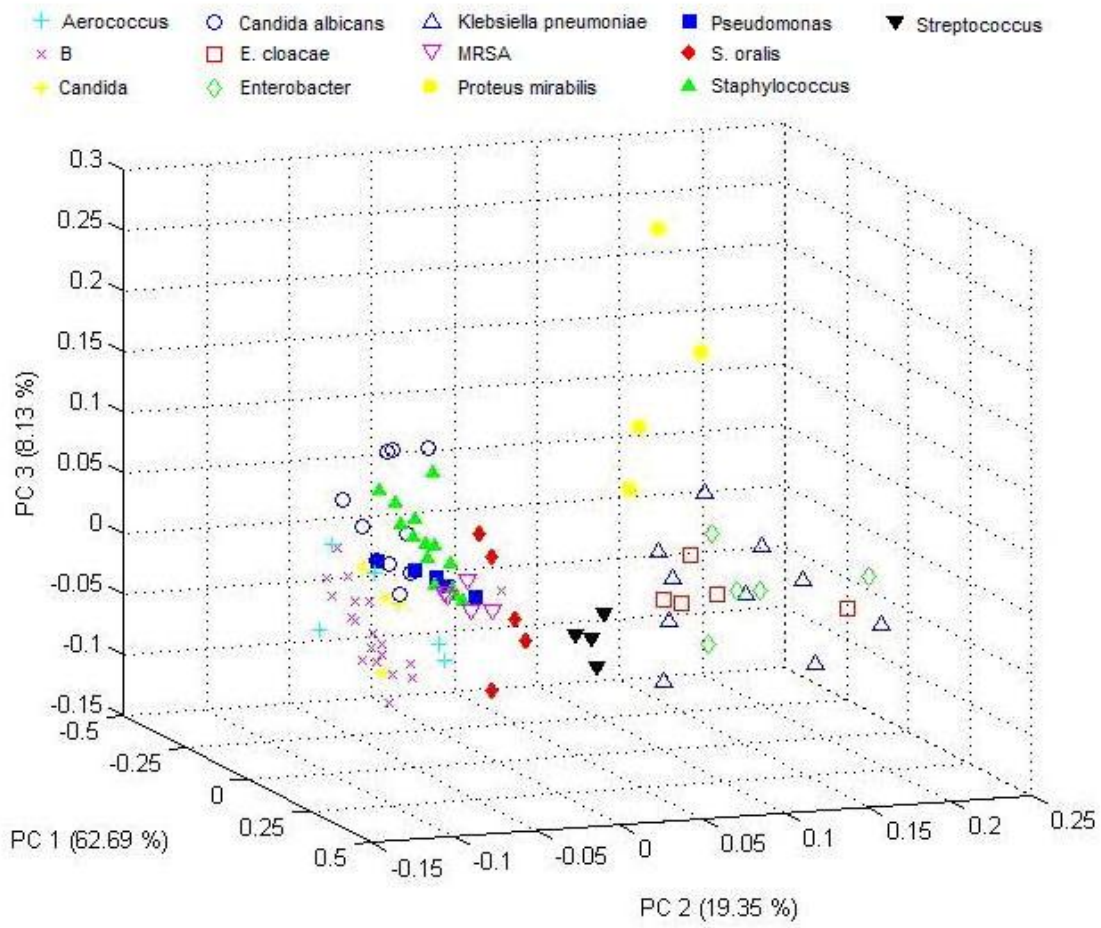



**Figure 4.1:** The 13 group PCA scores plot showing the discrimination between the various microbial species and the controls (B).

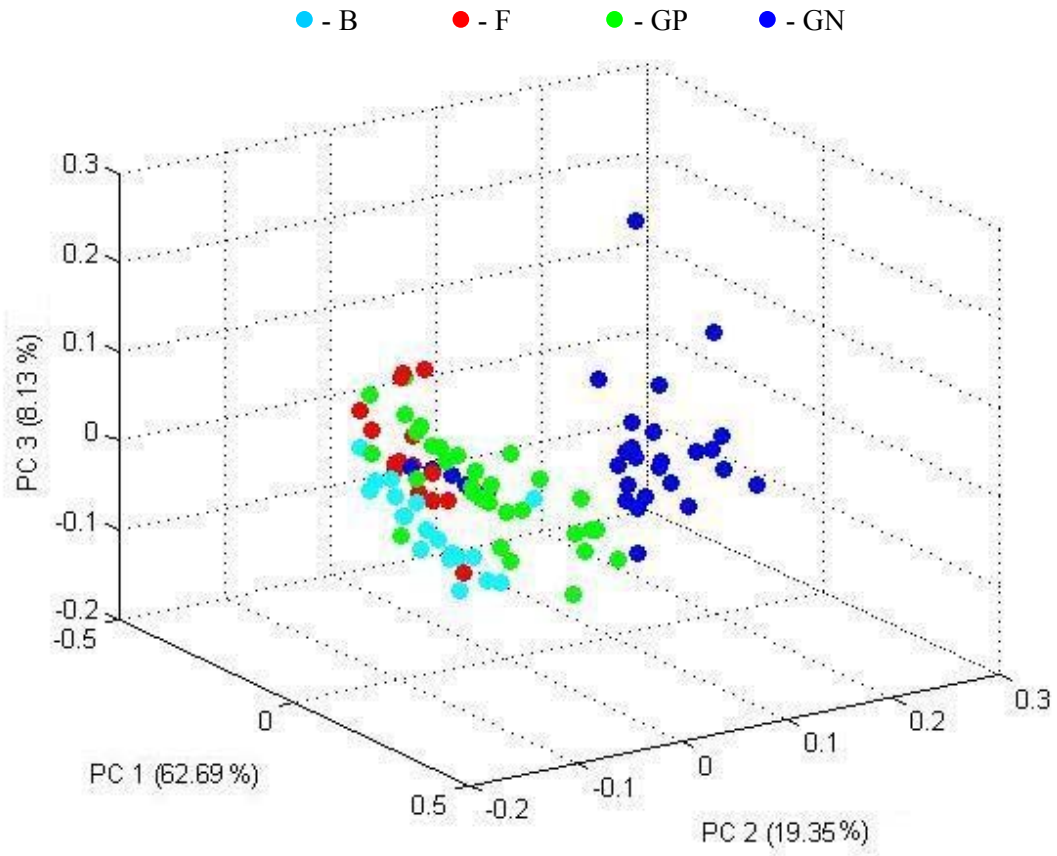



**Figure 4.2:** 3D PCA scores plot depicting segregation between the gram positive (GP), gram negative (GN), fungal (F) treatments and controls (B).

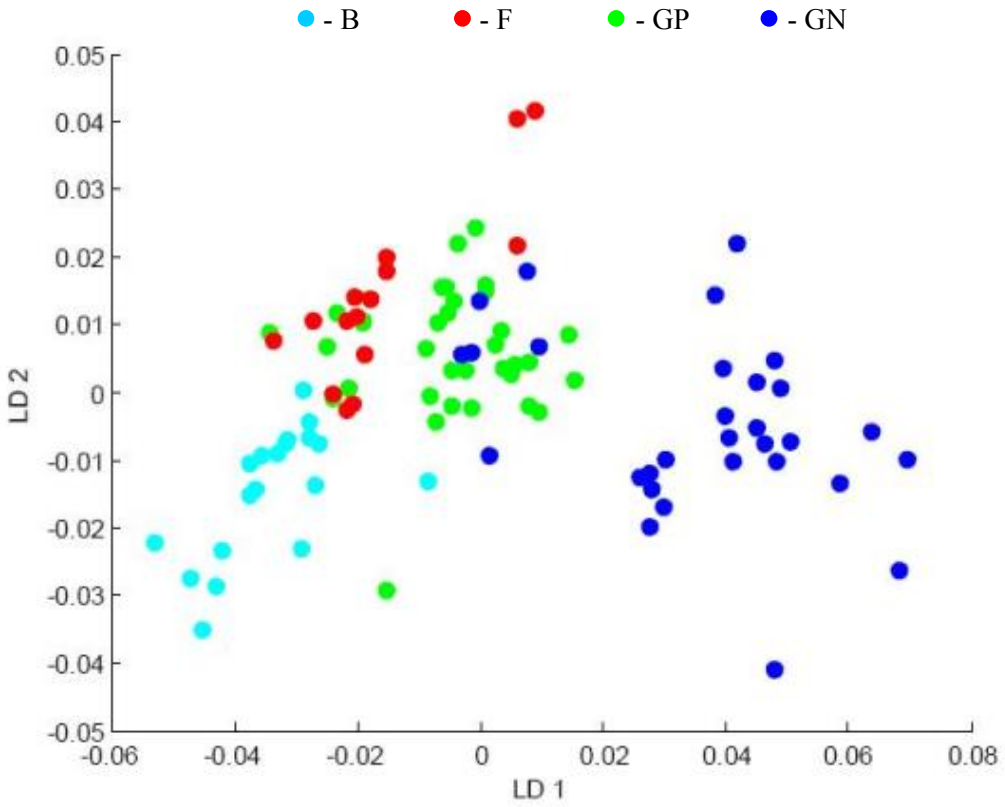



**Figure 4.3:** LDA of the four group model depicting segregation between the microbial species and the controls in nutrient broth.

(*Key:* B – Controls; F – Fungi; GP/GN – Gram positive and gram negative bacteria)

**Table 4.1:** Classification matrix of the four group model of microbial samples and controls having an 83% prediction accuracy.

(*Key:* B – Controls; F – Fungi; GP/GN – Gram positive and gram negative bacteria)

| | | Predicted Class | | | |
|---|---|---|---|---|---|
| | | GP | GN | F | B |
| Actual Class | GP | 25 | 0 | 8 | 1 |
| | GN | 5 | 24 | 1 | 0 |
| | F | 1 | 0 | 14 | 0 |
| | B | 1 | 0 | 0 | 18 |

**Table 4.2:** Distance measures between the microbial treatments and the controls based on the LDA model.

(*Key:* B – Controls; F – Fungi; GP/GN – Gram positive and gram negative bacteria)

| Class | GP | GN | F | B |
|---|---|---|---|---|
| GP | 0 | 9.11 | 3.40 | 7.74 |
| GN | | 0 | 15.30 | 22.95 |
| F | | | 0 | 8.54 |
| B | | | | 0 |

### 4.3.2 Analyses of clinical samples

Following headspace analysis of the clinical samples, the e-nose data was then correlated with growth information obtained from the hospital microbiology results. The data was again divided into the four groups as mentioned previously. From the 67 samples, only 62 samples were included in the analysis as five had no results from microbiology (Figure 4.4). However, a further ten samples, highlighted in the figure, were subsequently excluded from the analysis as they were considered to be outliers. The final results are shown in PCA scores plots (Figures 4.5 and 4.6) that depict the segregation of the clinical samples into the distinct groups. Subsequent discriminant analysis on the reduced data is illustrated in Figure 4.7. LDA indicated that 81% of the samples were correctly identified as those having microbial growth and no growth. Based on the four groups, the LDA model correctly classified 63% (n=52) of the clinical samples. Table 4.3 depicts the classification matrix of the LDA model. Furthermore, based on the e-nose sensor responses 12 of 31 samples were classified as infected, whereas these had no growth based on the microbiology results. Also, cross-validation studies resulted in a poor performance of 44% (data not shown).

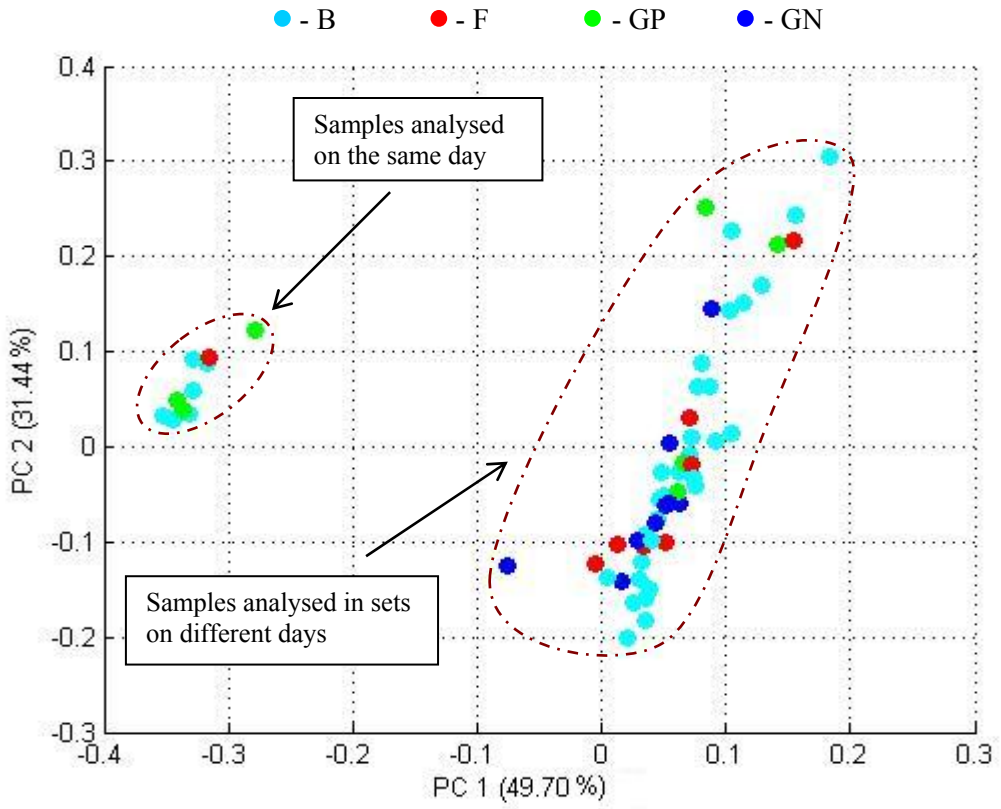



**Figure 4.4:** PCA scores plot of the four group clinical model indicating that a set of ten samples might be outliers.

(*Key:* B – Controls; F – Fungi; GP/GN – Gram positive and gram negative bacteria)

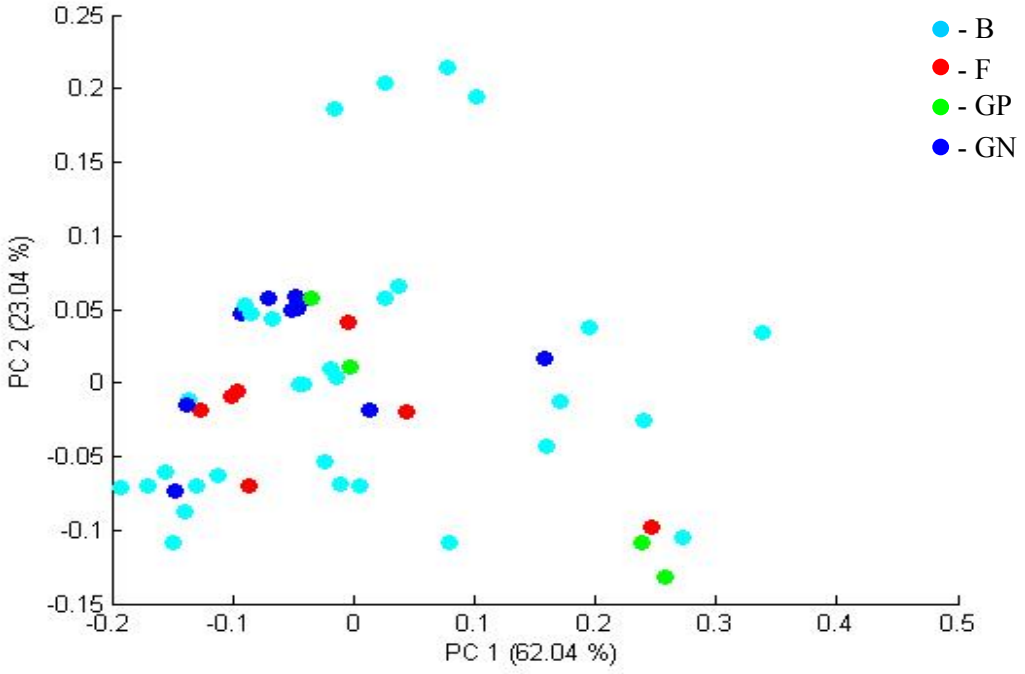



**Figure 4.5:** PCA map showing the classification of the reduced set of clinical samples (n=52) into their respective groups.

(*Key:* B – Controls; F – Fungi; GP/GN – Gram positive and gram negative bacteria)

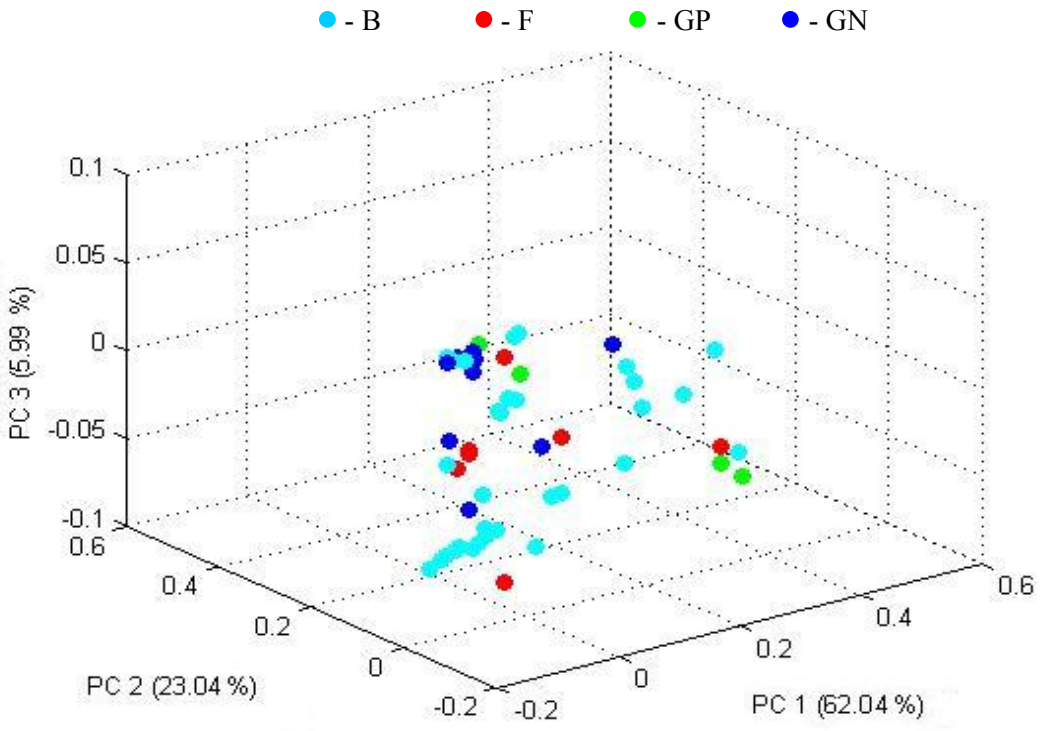



**Figure 4.6:** 3D PCA scores plot illustrating the classification of the 52 clinical samples into the respective four groups.
(*Key:* B – Controls; F – Fungi; GP/GN – Gram positive and gram negative bacteria)

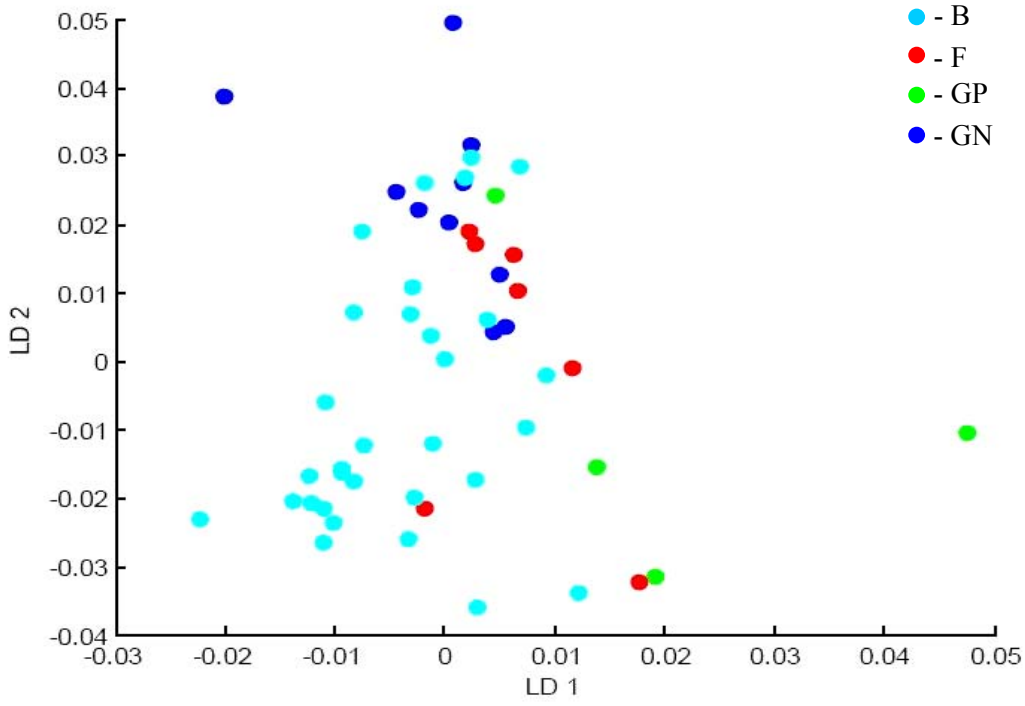



**Figure 4.7:** LDA of the 52 clinical samples depicting their differentiation into groups.
(*Key:* B – Controls; F – Fungi; GP/GN – Gram positive and gram negative bacteria)

**Table 4.3:** Classification matrix based on the LDA model of the clinical samples.

(*Key:* B – Controls; F – Fungi; GP/GN – Gram positive and gram negative bacteria)

| | | Predicted Class | | | |
|---|---|---|---|---|---|
| | | GP | GN | F | B |
| Actual Class | GP | 3 | 1 | 0 | 0 |
| | GN | 0 | 7 | 3 | 0 |
| | F | 1 | 1 | 4 | 1 |
| | B | 2 | 6 | 4 | 19 |

## *4.4 Discussion*

The potential of the e-nose to discriminate between microbial isolates from patients at risk or suffering from VAP as well as between actual clinical lavage samples was assessed. For *in vitro* studies, sixteen bacterial and yeast species were altogether analysed; but only a few species could be clearly distinguished from the rest, namely the *Enterobacteriaceae*. There also appeared to be a slight differentiation between the *Streptococcus*, *Staphylococcus* and *Candida* species. However, on the whole as the individual organisms could not be clearly differentiated, it was decided to opt for a four group system which enabled better discrimination. This was also seen when the supervised technique utilised resulted in 83% classification accuracy. Furthermore, it was observed that not all species actively grew in the medium (as the gram negative organisms) especially certain gram positive organisms and some *Candida* species which might explain why they cluster close to the uninoculated broth including the slight overlap between them. This implies a need for optimising the liquid medium to enable clear distinction between the individual species.

Another possibility for improving discrimination between the species could be by using lower concentrations of the organisms or shortening the overall incubation period. Boilot *et al.* (2002) reported the possibility of separating six species of bacteria in saline, in the form of pure lab cultures, responsible for eye infections using a conducting polymer e-nose and neural networks. They could not differentiate between the three concentrations of the individual bacteria but only when a single concentration was considered. In addition, they also distinguished two closely related *Staphylococcus* species, when considering four bacteria causing ENT infections. Although they made use of clinical samples, these were cultured on agar media overnight prior to analysis. This indicates that bacterial agar cultures probably produce more perceptible volatiles as compared to broth cultures, which was also suggested by Casalinuovo *et al.* (2006) for bacteria but not yeasts. There was no mention about the use of controls in either experiment, which may or may not have influenced the discriminatory capability of the bacterial species. Furthermore, if fewer bacteria were considered then even in the present study there might be a high probability of discriminating almost all species.

For e-nose analysis of the clinical lavage samples the four group model was used as it was thought to simplify their identification. Initial analysis revealed the presence of a set of samples that were considered outliers. There was reasonably good differentiation between the four groups of treatments, after the removal of ten samples. These samples could have had a change in their headspace during transport or storage or may also be due to some measurement variation. Additionally, some of these samples also contained mixed organisms based on microbiology and thus were removed. They could have been classified based on the dominant organism's growth but their effects would be unknown. In order to definitely interpret these kinds of samples, it would be interesting

to simultaneously carry out volatile profiling of such mixed cultures *in vitro*, in different proportions and then correlate them to the patient samples.

Correlating the microbiology results with e-nose sensor responses, classification was found to be more accurate in terms of growth and no growth (81%) when compared to the three microbial groups and no growth (63%). Additionally, the e-nose appeared to classify certain samples as infected even though the lab culture results suggested otherwise. On further analysis of this set of samples, it was found that the traditional CPIS system correlated with the e-nose prediction in 21 of the 31 samples. One of the most important factors that might influence such a classification was that most of the patients were administered antibiotics prior to sampling. This suggests that the drugs might be responsible for negative results by culture thereby affecting e-nose analysis. However there might be sufficient bacterial cells (following therapy) that produce some volatiles for the e-nose to detect and eventually classify them into certain microbial groups. Moreover, cross validation showed only a 44% prediction accuracy. This could be accounted for by the fact that only four patient samples were identified as gram positive. Nonetheless, further studies in this regard need to be carried out to establish this relationship; which could be based on *in vitro* antibiotic studies and analysing more clinical samples. This could also help in discerning if discrimination is based solely on the bacterial volatiles or host immune response to infection.

There have been a few other studies that have tried to use e-nose technology for discriminating VAP organisms (Serneels *et al.*, 2004; Moens *et al.*, 2006). Although both studies adopted the same experimental set-up i.e. using a ten metal oxide sensor e-nose and ten different microbes (*in vitro*), they used different analysis techniques and

reduced the total analysis time to 17 hours. Moens *et al.* (2006) reported 100% accuracy in prediction of the microbes using ANNs; however this was achieved by appropriate feature selection where the accuracy ranged from 77 to 86 to 100% depending on the treated or untreated data. Moreover, even in these studies there were no controls used in the form of uninoculated medium. In another recent study the breath of mechanically ventilated patients was analysed with a conducting polymer e-nose (Hanson & Thaler, 2005). The authors reported good correlation between the actual clinical pneumonia score (CPIS) and the e-nose predicted CPIS. They however, used only a small set of patients (19) as their culture data was available and did not mention the use of any control groups. A conclusion that the correlation obtained was purely on account of the microbes responsible for VAP or other contaminants or other substances that form a part of the instrument are thus debatable.

This technology has also been used for diagnosing other infections such as bacterial sinusitis, tuberculosis and urinary tract infections either *in vitro* or using clinical samples (Pavlou *et al.*, 2002a; Pavlou *et al.*, 2004; Thaler & Hanson, 2006). To enhance volatile production from clinical specimens Pavlou *et al.* (2002a, 2004) successfully spiked those using enzymes or nutritive broth. This approach could be useful in the present study to obtain clearer discrimination. Apart from identifying clinically important bacteria, e-nose technology has also been used in the food industry. For example to detect bacterial spoilage in milk matrices (Magan *et al.*, 2001) or *in vitro* detection of *Salmonella typhi* (Siripatrawan *et al.*, 2006).

This study examined the potential of the e-nose to identify volatiles arising from clinical bronchoalveolar lavage samples from patients at risk of VAP and correlated these

profiles to the cultured micro-organisms. This technique shows promise in allowing early diagnosis of VAP. Thus facilitating prompt administration of antibiotic therapy and thereby help in possibly reducing the associated morbidity. It also successfully differentiated between the main groups of organisms *in vitro*. However, more studies are required in order to improve the accuracy of this technique for clinical use.

# *Chapter 5*

# *VOLATILE PROFILES OF UPPER GASTROINTESTINAL MALIGNANCIES*

## *5.1 Introduction*

Cancer is a major health concern worldwide and is a result of one in four deaths in the U.S. and the U.K. The most commonly diagnosed types of cancer each year are those of the lung, colorectal, breast and prostate; which have both high incidence and mortality rates (Cancer Research U.K., 2007; Jemal *et al.*, 2007). Despite major advances in treatments in the form of new drugs or therapy, some of these cancers are still diagnosed at a late stage with poor prognosis. Therefore, a crucial factor to increase the survival rate of such patients post-treatment is detecting the disease in its infancy i.e. early stages.

However, early diagnoses may pose a problem for certain cancers because early on the disease is asymptomatic such as lung carcinomas or oesophageal cancers (Merck Manual, 1999). Thus new detection techniques have been and are being constantly developed, especially with a major research focus on disease markers. Previous studies have identified markers for lung, prostate, colon, pancreatic and breast cancers to name a few, mainly using immunohistochemistry or some molecular approaches on tissue or serum samples. Similar research on oesophageal cancer has also indicated the possibility of potential markers such as elevated levels of p53 proteins in precancerous/cancerous cells, p53 antibodies in sera of patients and the presence of either certain DNA alterations or cancer specific proliferating cell nuclear antigens in diseased individuals (Wang *et al.*, 1993; Lu *et al.*, 2003; Eisenberger *et al.*, 2006; Hammoud *et al.*, 2007a). Recently Hammoud *et al.* (2007b) made use of surface enhanced laser desorption/ionisation time-of-flight mass spectrometry (SELDI-TOF-MS) to identify patients with oesophageal adenocarcinoma based on their serum proteomic profiles. These techniques however are invasive due to biopsy or surgery and

blood withdrawal and can be expensive for clinical use especially the MS based techniques.

Non-invasive procedures are a relatively recent approach, mainly dealing with breath analysis and lately using tumour cell lines. Studies so far have however been on lung cancer and performed using new technologies such as e-noses to differentiate between diseased and healthy individuals based on markers in breath (Di Natale *et al.*, 2003; Chen *et al.*, 2005) or selected ion flow tube mass spectrometry (SIFT-MS) to detect the presence of VOCs *in vitro* in cell cultures (Smith *et al.*, 2003).

This study investigated the application of volatile fingerprints for the early diagnosis of and discrimination between the various forms of oesophageal cancer based on *in vitro* cell cultures using sensor array technology. It also aimed to identify compounds that may serve as potential markers of the disease. An attempt was also made to correlate the volatile profile patterns to *in vivo* situations by using clinical samples.

## *5.2 Materials and Methods*

### 5.2.1 Cell lines and media

The human cell lines used for the experiments were: HET-1A (ATCC No: CRL-2692), OE21 (ECACC No: 96062201), OE33 (ECACC No: 96070808) and CaCO2 (ATCC No: HTB-37). Further details regarding these can be found in Appendix A, A.3. These were maintained/grown in either RPMI-1640 or DMEM F-12 Ham (for the normal cell line) media (Invitrogen); each of which were supplemented with 10% foetal calf bovine serum (FCS), 500 µl penicillin/streptomycin, 1 ml amphotericin B and 1 ml glutamine per 500 ml media.

### 5.2.2 Cell culturing

All the cell lines were initially cultured in T25 tissue culture flasks (NUNC) in an incubator at 37°C with 5% $CO_2$. Once the cells reached 90% confluence, they were washed with phosphate saline buffer (PBS) and trypsinised (enzymatically dissociate the cells) using 5 ml of a 1x Trypsin (Sigma-Aldrich) solution per flask. Following 5 min incubation at 37°C, the mixture was transferred to a 15 ml centrifuge tube (Fischer) with equal volume of media to neutralise the enzyme. It was then centrifuged at 1500 rpm at 4°C for 5 min after which the pellet was re-suspended in 3 ml of media. This suspension was then equally apportioned to T75 culture flasks, to which 9 ml media was added and incubated as described previously. Media was changed every alternate day.

### 5.2.3 Differentiation of cell lines

The cells were scraped off the surface of the tissue culture flasks, once they reached 90% confluence, and 5 ml of the cell suspension was transferred to 25 ml vials and Universal bottles. Four replicates per treatment were set aside for an hour for headspace generation. Plain media were used as controls. The headspace was measured using the two sensor array systems (BH114, Bloodhound, UK and the NST 3320 - Nordic, Applied Sensors, Sweden) and samples were analysed in a random order.

Alternative sampling employed coating sterile e-nose vials with poly-L-lysine (Sigma) in order to enable the cell lines to adhere to the glass. The glass surfaces were coated with 5 ml poly-L-lysine solution for 10 min after which the solution was removed and the glassware allowed to dry overnight in the laminar cabinet. The screw lids and septa were UV-irradiated. The cell lines were then cultured as described previously and passaged to allow a total volume of 5 ml of cell suspension in the e-nose vials. They

were covered with a 0.45 μm nitrocellulose Millipore membrane filter (Sigma-Aldrich) - in order to allow gaseous exchange, and screw lids and incubated at 37°C with 5% $CO_2$. Three or five replicates per treatment were prepared. When the cells covered the lower surface of the vials, the media was changed; the membrane filters were replaced with septa and set aside for one, four or 24 hours for headspace generation at 37°C. The headspace was then analysed using the hybrid sensor array system (NST 3320, Applied Sensors, Sweden). In addition, the medium was transferred into separate vials, after which the medium alone, and the cells alone, were analysed as described earlier. These studies were repeated at least twice.

### 5.2.4 Detection of possible markers

100 ml medical flasks were autoclaved, coated with poly-L-lysine on one side and used for culturing cells (10 ml cell suspension) as illustrated previously. When the cells reached 90 to 100% confluence they were trypsinised, centrifuged with the pellet being re-suspended in 30 ml media and incubated for 24 hours at 37°C with three replicates per treatment. The headspace of the samples were then analysed for potential markers by using selected ion flow tube mass spectrometry (SIFT-MS).

The flasks were connected to the inlet capillary of the SIFT-MS instrument by piercing the septa with a needle connected directly to the instrument for analysis. The reaction of each SIFT-MS precursor ion ($H_3O^+$, $NO^+$ and $O_2^+$) with the sample was monitored for 90 seconds to generate mass spectra at m/z values between 10 and 160, using the full scan mode.

### 5.2.5 Clinical sample collection

Patients attending upper gastrointestinal (GI) endoscopy at the Gloucester Royal NHS Hospital were enrolled for an upper GI study (ethics approval in Appendix D, D.2). One part of the study involved sucking gas out of the oesophagus or stomach during the endoscopy from the suction port on the endoscope. These gas samples were trapped or sealed in air tight sputum traps and posted to the University for analysis using the hybrid e-nose (NST 3320 - Nordic, Applied Sensors, Sweden). The study was carried out blindly at the University on thirteen patient samples.

### 5.2.6 Data analysis

The sensor data collected was analysed by built-in software packages in both the electronic nose systems and Statistica 7 (Statsoft Inc.). Normalised data for divergence (indicating maximum step response) was analysed using XLStat (a Microsoft® Excel add-in) for the Bloodhound and mean-centred data for the response parameter was analysed using the NSTSenstool for the Nordic.

Multivariate statistics involving principal component analysis (PCA) and hierarchical cluster analysis (CA) were applied to the sensor responses to check for discrimination between sample treatments. The results were displayed in the form of PCA scores plots and dendrograms in order to identify any possible relationships between the cell lines.

In case of the patient samples, the PCA scores plots were analysed and the results were then correlated to the findings obtained during routine biopsy on the patient. This was done with the help of a clinician (based on information from the clinician) involved in the entire study based at the Gloucester Royal NHS Hospital.

## *5.3 Results*

### 5.3.1 Cell line differentiation

Headspace measurements of the cell lines using the conducting polymer (Bloodhound) system did not indicate any significant differentiation between the three cell lines (OE21, OE33 and HET1A) and the controls (the two media), even though the variance accounted for 99% of the data. The PCA scores plot shown in Figure 5.1 showed no specific discrimination although two clusters were observed. This was further supported by the dendrogram (Figure 5.2). On the other hand, the hybrid sensor system differentiated the two controls from each other and the treatments (Figure 5.3a) based on the first two principal components (PCs). The third PC however removed the segregation between the two controls when observed with the first PC but maintained the discrimination between the controls and the treatments (Figure 5.3b). The samples representing the normal cell lines, HET1A (N) appear to be clustering together and away from the remaining samples; but it might be the effect of the different medium used for its culture. In either case, approximately 97-98% of the data was represented by the variance.

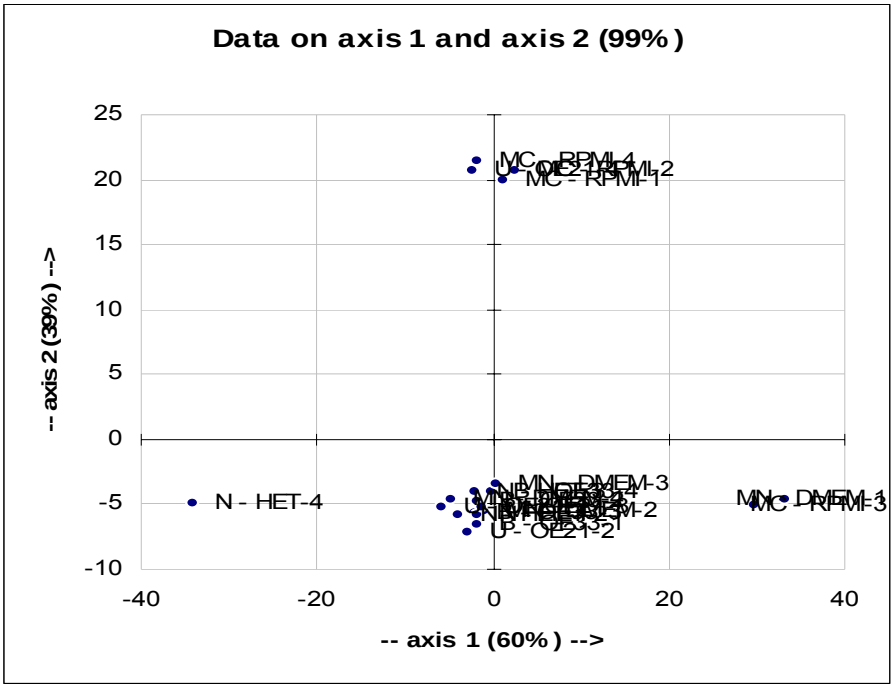



**Figure 5.1:** Data from the conducting polymer sensor array in the form of a PCA map showing no discrimination between the controls and cell lines.

(*Key:* N–HET1A; U–OE21; B–OE33; MN–DMEM medium; MC–RPMI medium)

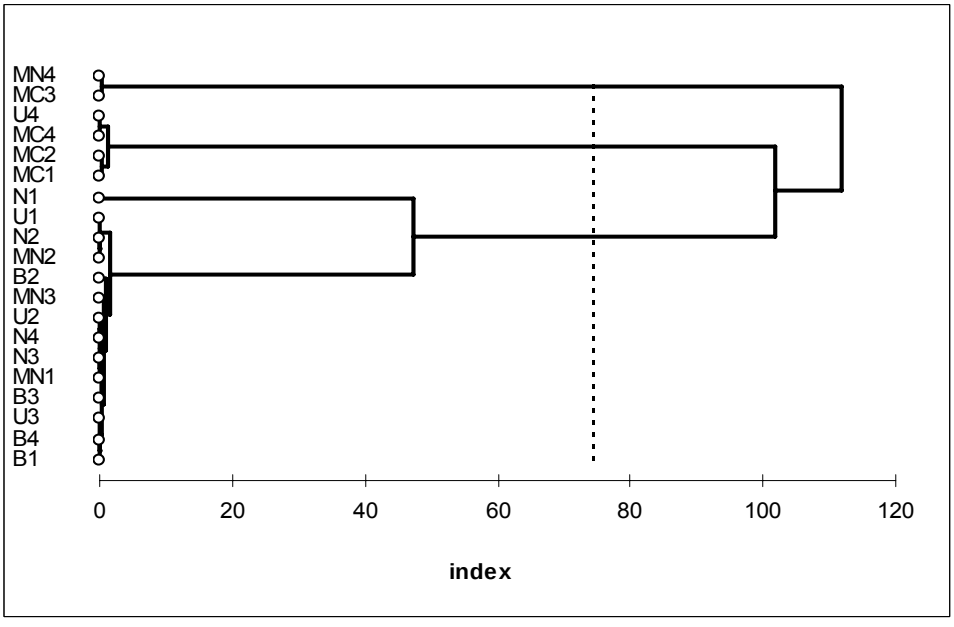
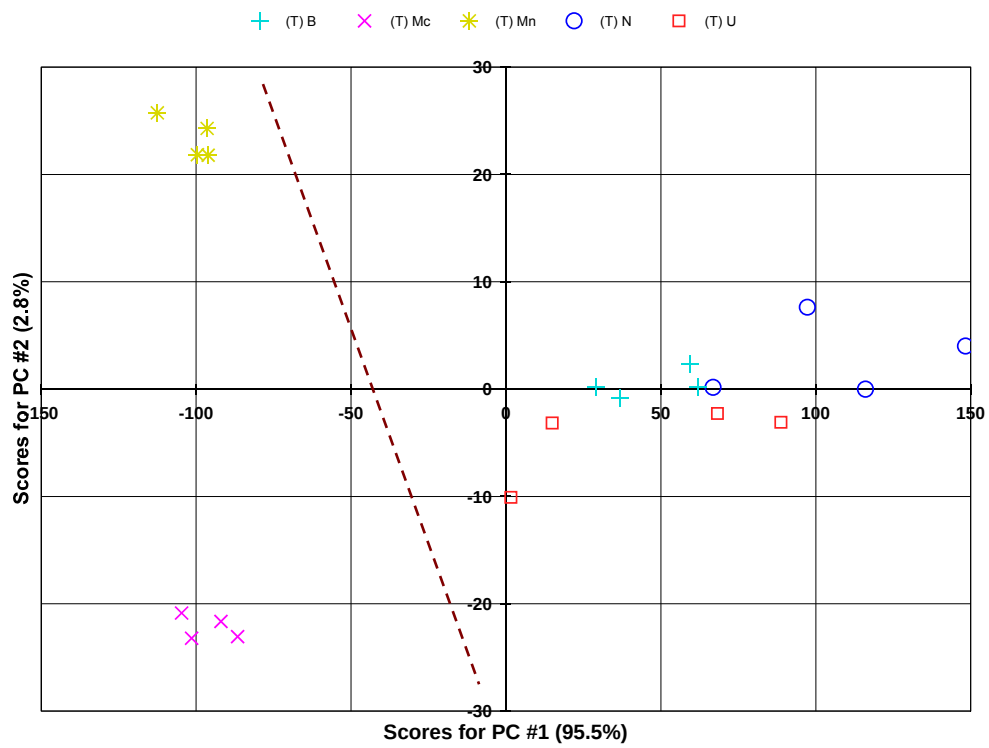



**Figure 5.2:** Dendrogram from the BH114 e-nose indicating no differentiation between samples including controls.

(*Key:* N–HET1A; U–OE21; B–OE33; MN–DMEM medium; MC–RPMI medium)

(A)




(B)

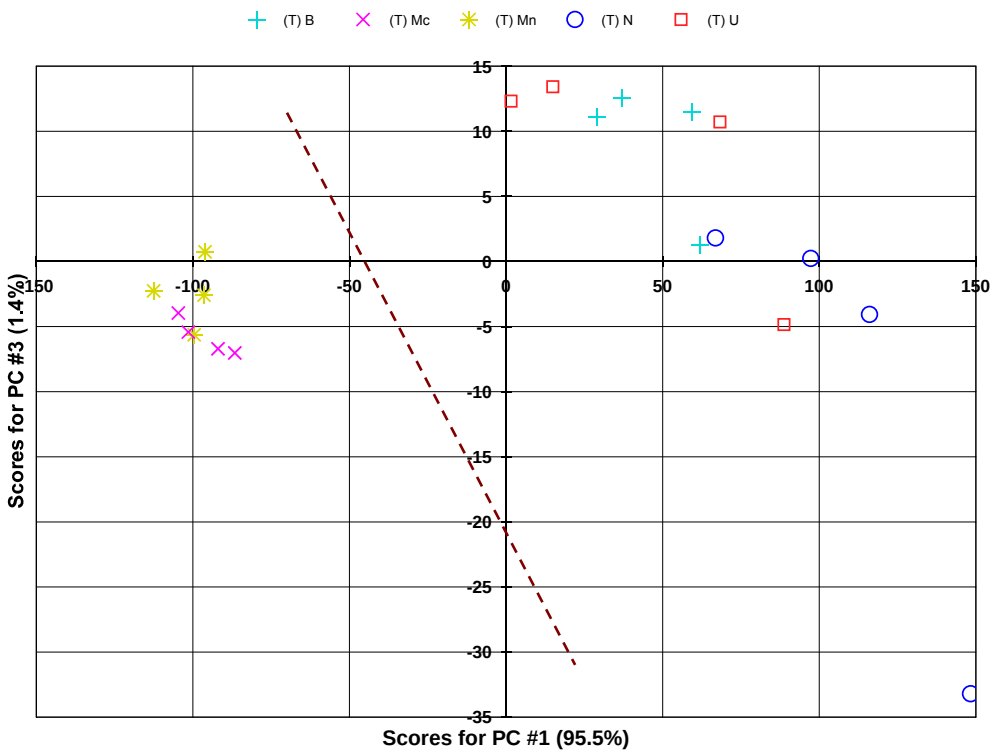



**Figure 5.3:** Discrimination between the controls from each other and treatments seen in PC1 vs. PC2 (A) and no discrimination between the two controls in PC1 vs. PC3 (B) with data from the hybrid sensor array.

(*Key:* N–HET1A; U–OE21; B–OE33; Mn–DMEM medium; Mc–RPMI medium)

Subsequent experiments were performed using a single medium with the cells being grown in glass vials itself. Analysis of the headspace of the cells growing in medium after one hour incubation at 37°C, suggested no discrimination between controls and treatments based on the PCA scores plot. Cluster analysis, using Euclidean distance and Ward's linkage, confirmed this (Figure 5.4). An extended incubation time of four hours for headspace generation also resulted in the same outcome. Similarly, no differentiation was observed when just the headspace of the cells without the medium were analysed. In case of only the media from the cells being analysed, the controls (blank media) appeared to separate from that of the others as seen in Figure 5.5, but was not sufficiently distinct.

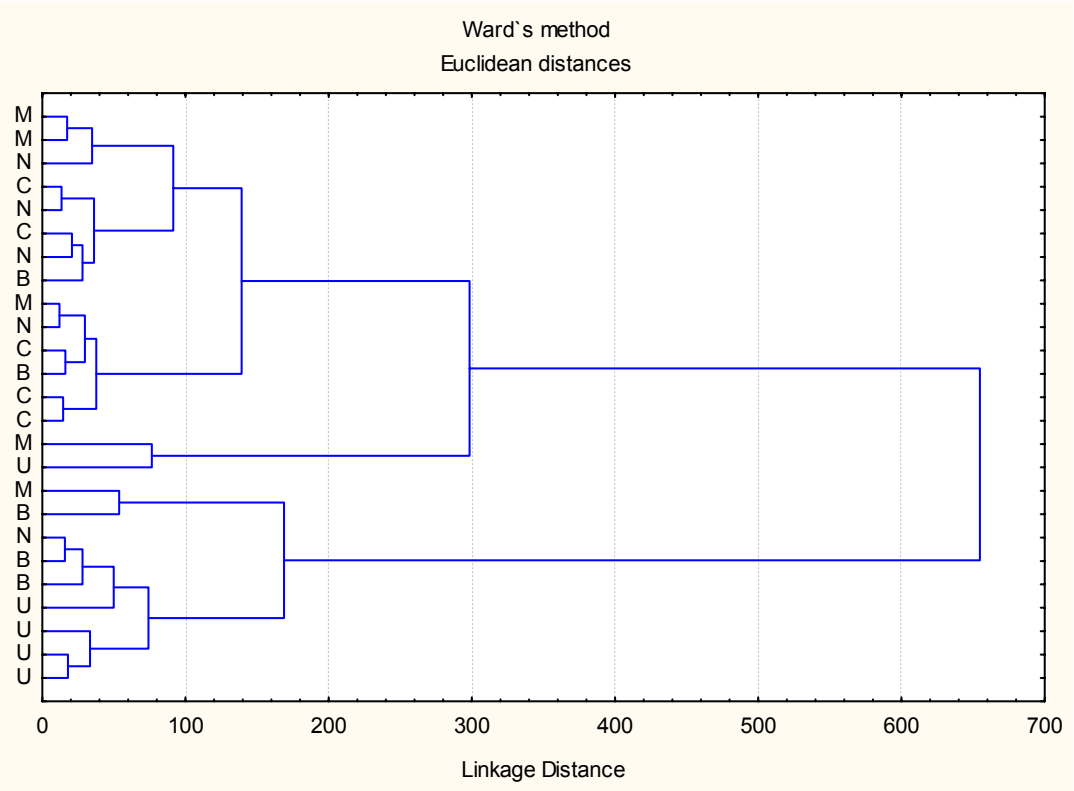



**Figure 5.4:** Cluster analysis on e-nose data from cells in medium depicting no differentiation between the controls and cell lines after 1 hour headspace incubation. (*Key:* M – RPMI medium; C – CaCO2; N – HET1A; U – OE21; B – OE33)

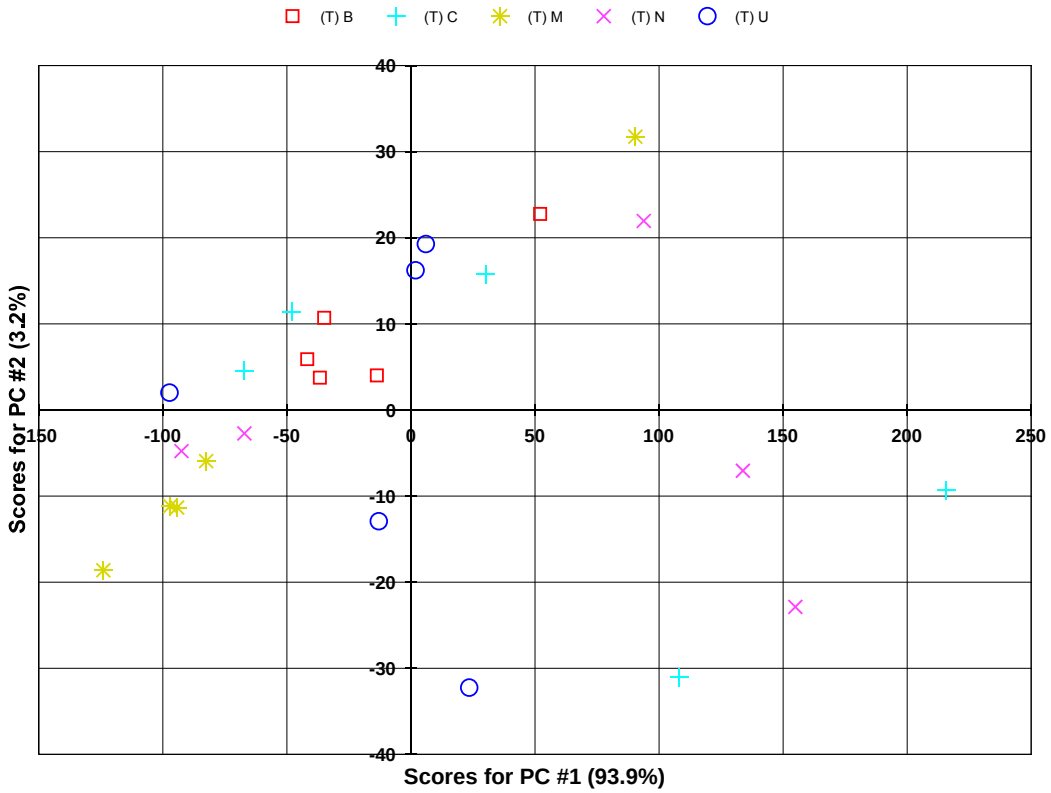



**Figure 5.5:** PCA scores plot indicating no distinct separation between only the media of the cells and the control medium after four hours incubation for headspace.

(*Key:* Media from HET1A (N), OE21 (U), OE33 (B), CaCO2 (C) cell lines and control medium (M))

However, when the headspace of the cell cultures in media were analyzed after 24 hours incubation at 37°C not only could the controls (pure medium) be differentiated from the other treatments, but also the OE21 samples were clearly separate (Figure 5.6). The normal and OE33 samples could not be distinguished and were clustered together, but those belonging to CaCO2 even when considered with the first three PCs (accounted for 99.5% of variance) were only barely distinct from the normal and OE33 cluster (Figure 5.7). Figure 5.8 depicts the clusters formed by all the cell culture treatments and the medium in the form of a dendrogram.

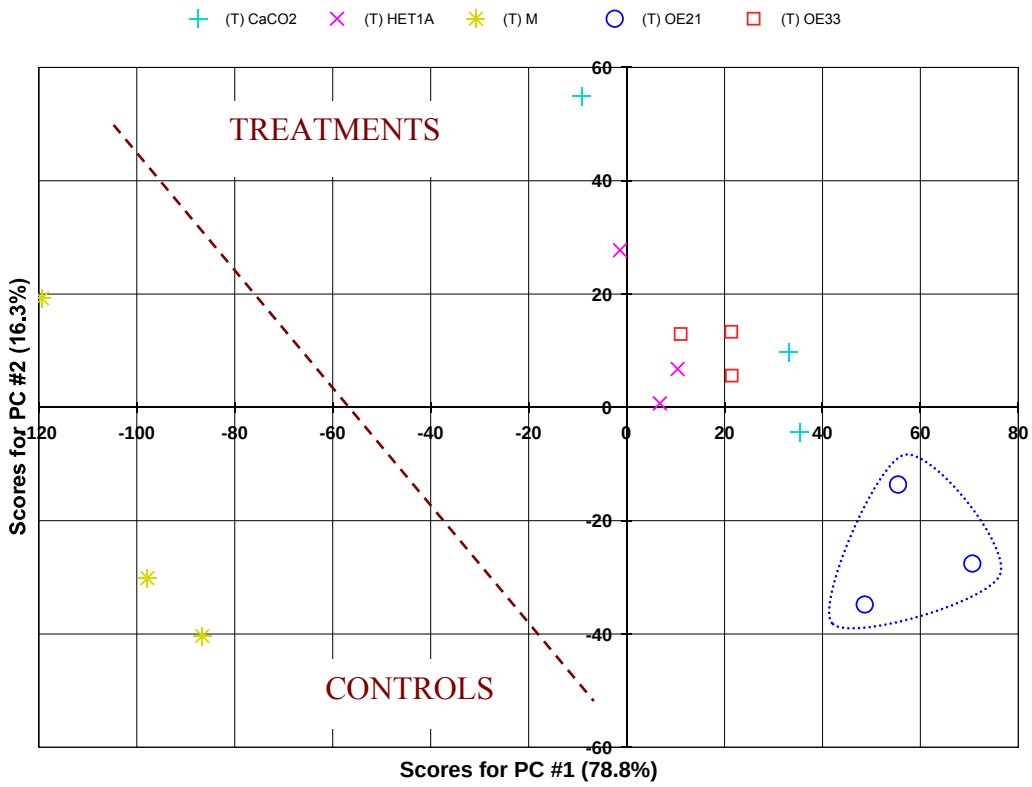



**Figure 5.6:** Controls (M) and OE21 cells being clearly segregated from the other cell cultures after 24 hour incubation for headspace generation in a PCA plot.

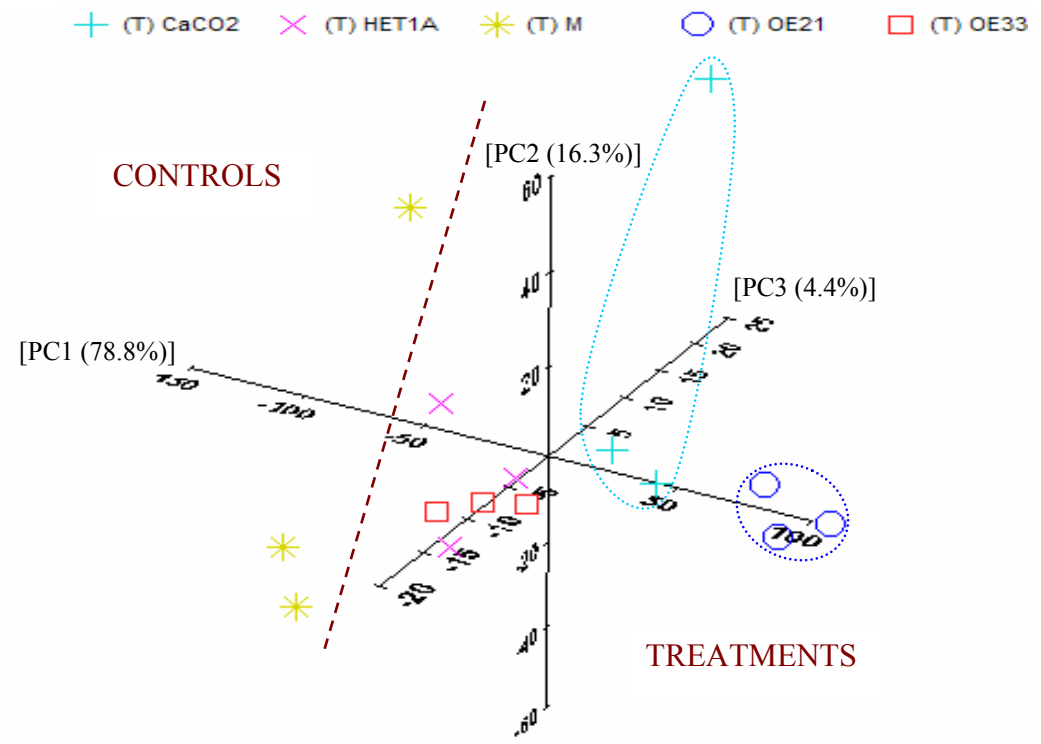



**Figure 5.7:** 3D PCA plot depicting clear separation between controls (M) and OE21 cells, but slight separation of CaCO2 cells from the other samples.

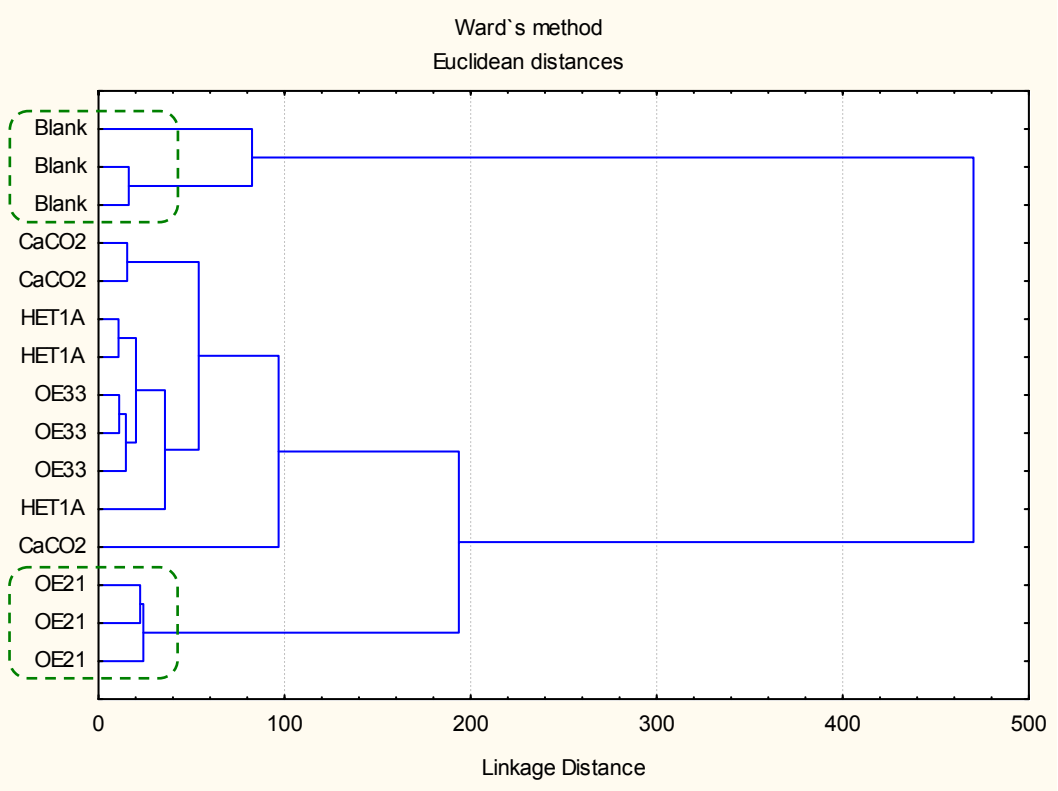



**Figure 5.8:** Dendrogram of the cell cultures after 24 hour incubation depicting clear clusters of controls (Blank) and OE21 cells.

### 5.3.2 Detection of potential markers

Headspace analysis of the various cell lines and controls by SIFT-MS did not indicate the presence of specific markers or identifiers, but detected very few analytes. Ammonia was found to be present in higher amounts in the controls (medium) than in the cell lines. The acetaldehyde concentration was higher in the cell lines than in the medium, as was the case with a compound at m/z 93 which could be toluene but due to its trace quantity difficult to identify. Methanol also appeared to be present in the CaCO2 cell lines in trace amounts. Two unidentified compounds at m/z 44 and 98 were present in low concentrations in the cell lines and absent in the controls, with the former probably being slightly higher in the CaCO2.

### 5.3.3 Clinical sample analysis

The clinical samples containing gases trapped from the upper GI tract were analysed using the metal oxide-metal ion e-nose. Based on the post-endoscopic information provided by the clinician, each of the samples were assigned respective classes based on their pathologies. The first set included six patient samples, data analysis on which indicated that no obvious clustering of the pathologies was observed. However, when all the thirteen clinical samples data were analysed simultaneously by means of PCA, no specific pattern was achieved to discriminate the pathologies (Figure 5.9).

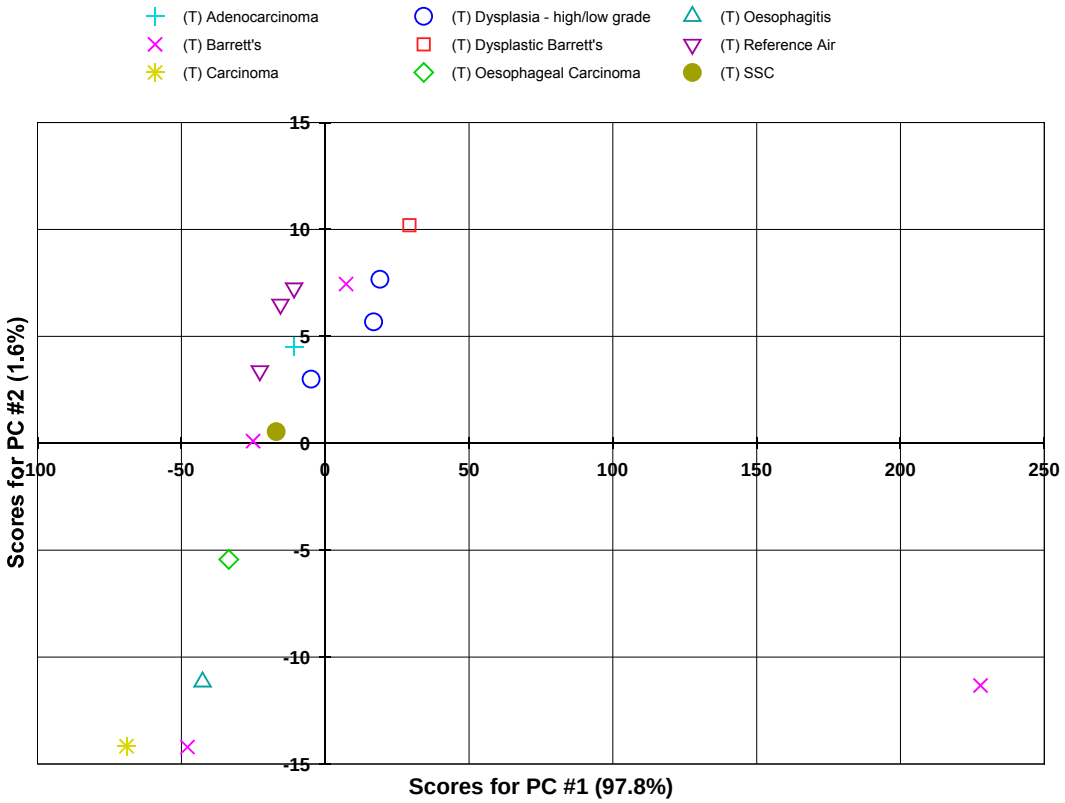



**Figure 5.9:** PCA map of the clinical data depicting no discrimination between the various upper GI pathologies.

## *5.4 Discussion*

There have been many reports of e-nose systems with different types of sensor arrays outperforming others based on a diverse range of applications. Similarly, in this study the hybrid e-nose (metal oxide-metal ion) appeared to be better than the conducting polymer system, even though the former only distinguished between the controls (plain media) and treatments (cultures of oesophageal cell lines). Multivariate analyses on the data from the latter did not result in any sort of distinction, albeit the variance accounted for was extremely high. To my knowledge this is the first study using e-nose technology for discriminating between human oesophageal cancer cell lines. Only recently, a preliminary study has been attempted to differentiate between six different human lung carcinoma cell lines using a conducting polymer e-nose (Gendron *et al.*, 2007). The authors used saline suspensions of the cancerous and normal (fibroblasts and smooth muscle) cells and could differentiate between them in two separate sets each with three cell lines. However, their results might be altered if all the cell lines were analysed together. In addition, they did not use saline as a negative control. Control media could have a significant impact on the discrimination obtained. In the present study, a medium effect was observed which was due to the higher response of the $CO_2$ sensor for the normal medium (DMEM) seen on the second PC, when the loadings were examined. This could perhaps be on account of the constituents of the medium.

Other e-nose based cell line studies have mainly involved industrially important animal cell lines such as the Chinese hamster ovary (CHO) cell line that are used for the production of pharmaceutical products e.g. recombinant proteins. These studies mainly deal with on-line bioprocess monitoring and detection of microbial contaminants (Bachinger & Mandenius, 2000; Kreij *et al.*, 2005). The other medical applications have

included breath analysis for diseases or detecting infections or organ disorders (Fend *et al.*, 2004; Pavlou *et al.*, 2004; Dutta *et al.*, 2005).

The subsequent selection of a single medium for culture was done in order to resolve the potential effects of the separate media. Additionally, another cell line representing colorectal adenocarcinoma was included in the study. It was observed that initial lower incubation periods for headspace equilibrium did not lead to marked differentiation between the cell lines and the controls, when grown in the vials. However, a 24 hour incubation period prior to headspace analysis resulted in definite segregation between the controls and at least one cell line (OE21). This could possibly be because more time is required for the compounds/metabolites released by the cells to move into the headspace. This may also be because the medium of the cells was changed before setting aside for generating the headspace resulting in insufficient volatiles being present. Another explanation could be that the cell numbers were relatively low which lead to lower production of organic compounds (probably as the rate of cell generation for the various cell lines is not uniform) or cell viability was affected. In support of the former, previous *in vitro* studies on lung cancer cells have indicated that the amount of compounds in the headspace is directly proportional to cells (Smith *et al.*, 2003). These could possibly be overcome by measuring the culture suspension without changing the medium or using a more concentrated cell suspension; that might also enable differentiation between the other cell lines.

Apart from only qualitative differentiation, the SIFT-MS technique further enabled the search for potential markers for this condition. However, not many organic compounds were identified by this process. This might be because the headspace of the samples was

not sufficiently concentrated. That in turn could also be the result of a lower concentration of cells unable to produce sufficient volatiles for detecting specific identifiers, if not markers. Smith *et al.* (2003) reported the production of acetaldehyde in the headspace of two lung cancer cell lines *in vitro* using this technique. They simultaneously indicated that the production of the compound was affected when a cell line was treated with a medically used cytotoxic drug. Other *in vitro* studies have indicated the presence of elevated levels of formaldehyde in a variety of human tumour cell lysates – breast, cervical, leukaemia, when treated with anti-tumour drugs (Kato *et al.*, 2000; Kato *et al.*, 2001). Formaldehyde was also shown to be present in higher concentrations in the urine headspace in patients suffering from bladder and prostate cancer than in healthy individuals (Španěl *et al.*, 1999).

Markers for upper GI malignancies have long been researched, nevertheless none have been put to practical use for the purposes of screening. Biopsied tissue and serum from patients were shown to indicate elevated levels of p53 proteins based on immunohistochemical staining and immunoassays for oesophageal squamous cell carcinoma (Ralhan *et al.*, 2000; Shimada *et al.*, 2000). It has also been suggested that these serum antibody levels could be used for monitoring the treatment of the disease. Recently, Hammoud *et al.* (2007a) reported the presence of a possible marker - cancer specific proliferating cell nuclear antigen (csPCNA) only in adenocarcinoma tissues that could enable identification of invasive oesophageal cancer from varying forms of dysplasia and no cancer. The authors also developed a specific antibody against the antigen. Proteomics has lately also gained popularity, where mass spectrometry has been coupled to other techniques to differentiate normal individuals from those with cancer based on either their serum protein profiles (Hammoud *et al.*, 2007b) or up/down

regulation of specific proteins present only in cancerous tissues (Zhou *et al.*, 2002; Nishimori *et al.*, 2006).

Clinical samples were also utilised in this study to further investigate the potential of the e-nose to differentiate between the various oesophageal tumours. In the thirteen patients that were studied, there was no specific pattern of discrimination that was observed. It could probably be on account of the sampling technique, where endoscopy was utilised to trap the gas. One more rationale could be that the samples were not analysed instantly on site, and the transport and storage might have changed the headspace. However, it must be noted that the sample size was extremely small. Furthermore, due to certain problems associated with endoscopic gas sampling, further clinical samples were not collected. Perhaps direct breath analysis of such patients might be an alternative that could yield better results. Breath has been previously studied using e-noses with gas sensors for identifying patients with lung cancer from healthy subjects (Di Natale *et al.*, 2003; Chen *et al.*, 2005); but these authors built their instrument based on sensors specific to alkanes and benzene derivatives that have been shown to be markers of the disease. Therefore, facilitating distinction between healthy individuals from those that were diseased.

This study is the first of its kind to make use of volatile profile patterns of tumour cell lines for differentiating between the various forms of oesophageal cancer from normal cells *in vitro*. Although *in vitro* studies show some promise, in case of clinical samples alternate methods need to be explored. There is definite advantage for this technique in a clinical setting for fast, early and probably non-invasive diagnosis. It shows potential for such use, but requires significant further investigations to draw definite conclusions.

# *Chapter 6*

# *INTEGRATIVE DISCUSSION*

The present chapter highlights some of the common features that were encountered during the process of the studies presented in this thesis and attempts to describe the steps taken or suggest any alternative precautionary measures. The main aspects comprised sampling issues – their collection, preparation for analysis or even the representative size; certain aspects of data analysis or in relation to the instruments utilised.

## *6.1 Sampling*

Presentation of standardised samples and good replication along with randomisation is important in obtaining useful results using electronic nose systems or any other type of analyses for that matter. In certain cases some sample replicates were considered 'outliers' as they either fell into different groups or deviated substantially from their respective class after multivariate analysis. This could possibly be either on account of the screw cap on the e-nose vial being loose leading to outside air altering the headspace or too little/too much growth of a specific replicate in case of both microbial species and cell line samples. Removal of such outliers considerably improved the variance and thus the discrimination between the microbial species (for e.g. from ≈ 73 to 83% for VAP clinical samples). Nevertheless, care must be taken whilst identifying outliers because it might just be that the sensitivity of the sensors has gradually changed over time causing a drift and thus varying responses. There might also be a slight possibility of genetic drift, probably due to repeated sub-culturing or species attenuation causing the change.

Furthermore, the type of medium also appeared to affect the results. It was observed that in broth cultures, especially the fungal cultures, replicates showed more variation than in agar. This could be on account of relatively tighter vial lids preventing aerobic

respiration or insufficiently homogenised initial mixtures causing certain samples to contain relatively more CFUs. Additionally, a depletion of nutritional content over time in liquid broth (based on the mean $CO_2$ response) could also have affected results. Casalinuovo *et al.* (2006) also reported similar issues regarding volatiles from bacterial broth cultures. Thus, further investigation is required for liquid medium optimisation since it might prove to be feasible for clinical specimens.

Nutritional constituents that make up the media also appeared to play a role in the volatile generation. This effect could be observed when two different agar media were used for discriminating the *Trichophyton* species suggesting that specific media could be used to enhance volatile production. Pavlou *et al.* (2002a; 2004) made use of complex media with supplements or enzymes to facilitate volatile production from the clinical samples.

Besides these, the low concentration of volatiles in certain types of samples could also affect their discrimination. For example, samples where low levels of detection are essential (microbes) or where the sample availability is limited - in terms of the amount of sample being scarce (cell lines or clinical samples). In these instances preconcentration of the samples prior to exposure to the sensor array could enable detection of small amounts of volatiles. Mostly such research has been performed with GC-MS type methods. However, recent studies have made use of MS based e-noses or piezoelectric based sensor arrays along with SPME fibres for preconcentration of volatiles based on foods, beverages or human breath (Marsili, 1999; Schaller *et al.*, 2000; Pearce *et al.*, 2002; Marín *et al.*, 2007).

## *6.2 Sensors*

Commercially available e-noses have a broad range of specificity due to the nature of the sensor arrays as well as the number of sensors that make up the array, i.e. they are generic in nature. Therefore, it is of utmost importance that the sensors chosen are suitable for the application in question. In this study, it was found that the hybrid metal oxide sensor array was more suitable than the conducting polymer sensors.

Occasionally it was found that the first sample to be analysed might not be a representative of the replicates in that set, since it responded variably to some sensors. This could be on account of the instability of the sensors or the instrument warming up after being idle. It is however, not necessary that all sensors give meaningful information. They might rather be contributing to background noise or produce very low signals rendering them insignificant. Hence, suitable sensor selections by means of PCA loadings improved discrimination and overcomes the effect of noise.

This raises another issue regarding the long term stability and reproducibility of such sensors. On the whole however, the hybrid sensors used during this research showed good reproducibility and gave consistent results. Although towards the end of the study some of the sensors did appear to show signs of slight drift (Appendix B, B.2) which indeed highlighted one of its main drawbacks. It could be on account of sensor material ageing or poisoning or saturation of the sensors due to exposure to excessive amounts of a specific volatile compound. The latter could possibly explain the inability of discriminating low thresholds ($10^{1-2}$ cells $ml^{-1}$) of the fungal species. The excessive growth of the higher inocula after 96 hours might have saturated the sensors masking the effect of the volatiles, if any, at the lower inoculum levels.

Nevertheless, in order to alleviate or remove the effect of sensor drift, studies have suggested use of external calibration samples along with the application of certain mathematical algorithms, whereby the drift direction from the external calibration sample is removed from the remaining samples (Artursson *et al.*, 2000; Haugen *et al.*, 2000). This approach could prove beneficial for future use on the e-nose systems used in this study. Furthermore, recently researchers have reported the use of signal filtering methods to remove drift (Zuppa *et al.*, 2007). Since the field of sensor technology is constantly evolving, there is definite scope for improvement.

### *6.3 Multivariate analyses and computational prediction*

The ability to classify unknown samples into specific groups is a more attractive prospect for the food quality and medical sectors, apart from just the normal identification or discrimination of samples. Multivariate techniques involving PCA, DFA or PLS were normally used for making models for this purpose, but Goodner *et al.* (2001) warned that if not used with care, these methods could easily lead to erroneous results. Subsequently, the use of more robust and non-linear methods such as ANNs gained popularity for building such classification models.

However, an important feature that could hinder the results is the sample size. It is extremely important that samples are well represented and are sufficient to allow appropriate training followed by validation or testing of the network model. In this thesis because of the relatively small sample size, for ANN analysis the 'leave one out cross validation' approach was administered. It involved individually testing every sample against the trained network. Furthermore, the ratio of samples and variables could also lead to incorrect classifications. In general the number of samples must be

much higher than that of the variables. Another constraint with neural network models is over-fitting where it just memorises the training data. This implies high accuracy during training but results in poor validation and thus poor generalisation i.e. the inability to correctly predict an unknown.

Integrating artificial neural networks with evolutionary methods such as genetic algorithms further serves to enhance the performance of the networks. This form of evolutionary computation enables feature selection, structure optimisation and improves the overall efficiency of ANNs. Thus enhancing their discriminatory and classification power. This approach has been successfully used by Pavlou *et al.* (2000; 2002a; 2002b; 2004) for differentiating various bacteria using e-nose data.

## *6.4 (Bio)-marker identification*

Identifying markers, more specifically biomarkers, would significantly aid the process of diagnosing medical conditions or detecting the presence or absence of certain micro-organisms to administer drugs. However, it is not always possible to find markers as these products depend on the metabolism of either the host (can also be the host's immune response) or the invading microbe or possibly a combination of both. In the present study it was found that certain compounds were present in higher concentrations in some organisms than in the others. Perhaps by using a combination of these detectable compounds might help form a 'marker pattern' that could be correlated to the e-nose volatile profiles for individual microbes.

Identification of marker compounds is predominantly done by means of GC-MS techniques. However, these are not practical for routine use in clinics. Therefore, once

specific markers are identified - e.g. those belonging to specific microbial species or specific cancers (or stages thereof) - based on the current detection methods it increases the probability of building smaller, portable e-nose instruments with fewer sensors. Thus suggesting there is potential for regular clinical utilisation including as a screening tool.

### *6.5 Real-time analysis and monitoring*

The major challenge of e-nose technology is its eventual use in the desired field. Based on the literature and present studies, the e-nose is a good instrument for obtaining simple yes-no answers but how reliable would it be for the next level? In case of the research in this thesis, it has been successful for most species level discriminations. The ultimate goal however, would be analysing the clinical samples in real-time with an application specific portable instrument. It could lead to the standard laboratory protocols or other routine invasive diagnostic procedures being completely bypassed. This would enable rapid if not instant diagnosis, facilitating the clinician to take prompt action – whether that is prescribing appropriate medication or initiating other forms of treatment. It might also form the basis for initial screening of diseases and possibly checking for building drug resistance, especially in microbial infections. Another asset of a portable machine would be the additional ability to use it for monitoring the progress of diseases and individuals, and thus truly moving it from *'bench to bedside'* in relevant scenarios.

# Chapter 7

# CONCLUSIONS & SUGGESTIONS FOR FUTURE WORK

## *7.1 Conclusions*

The main aim of the present research was to use volatile profile patterns in order to diagnose different kinds of diseases in their infancy, which in turn would alleviate or perhaps even eliminate the need for the traditional 'gold-standard' and invasive techniques. This section highlights the main points and inferences from the various phases carried out in this research.

A. Dermatophytes:

- From the two different e-nose systems that were compared, the hybrid (metal oxide-metal ion) sensor array system proved to possess better discriminatory capabilities than the conducting polymer sensors, over a period of 72-96 hours of fungal incubation even after using an alternative sampling method for the latter.
- There was clear differentiation between the four pathogenic *Trichophyton* species (*T. mentagrophytes, T. rubrum, T. verrucosum* and *T. violaceum)* on both solid media within 96 hours of incubation. Liquid culture studies gave similar results but were not as well defined as those from the agar. With the starting inoculum of $10^{5\text{-}6}$ spores $ml^{-1}$ in each instance.
- The threshold for detecting the sensitivity of *T. mentagrophytes* and *T. rubrum* in both forms of media was found to be an initial concentration of $10^3$ CFU $ml^{-1}$ after 96 hours, although for broth cultures once again the clusters were not as distinct as for the solid media.
- In terms of intra-strain comparisons, it was found that the strains of *T. rubrum* were more different from each other than the *T. mentagrophytes* strains on comparing their volatile fingerprints respectively after 96-120 hours. These

similarities and differences were also observed when correlated with the respective growth rates.

- A clear discrimination between the fungi responsible for animal (*M. canis*) and human (*T. mentagrophytes* and *T. rubrum*) infections was also found to be possible based on the volatile profiles, within 96-120 hours.
- Interestingly, large concentration of ammonia was detected by SIFT-MS to be present in the fungi, especially in *T. mentagrophytes*. Other compounds of interest were traces of dimethylamine, formaldehyde and hydrogen cyanide present only in the fungi. Although, no specific markers were identified.
- GC-MS detected the presence of a few compounds in the fungi that were absent in the controls such as methoxybenzene (high in *T. mentagrophytes*), 1-Octen-3-ol and 3-Octanone (the last two being larger amounts in *T. rubrum*). These could help serve as potential identifiers.
- The simulated predictive models successfully classified the samples into their respective classes with high accuracy. It was also seen that the use of fewer sensors improved the classification ability of the network model.
- The antifungal agent, itraconazole, almost completely inhibited the growth of the two dermatophytes (*T. mentagrophytes* and *T. rubrum*) at 2 ppm with its effect being more pronounced at 25 than at 30°C.
- The antifungal treatments were clearly differentiated from those without the antifungal at both temperatures, with the two non-treated fungal species also being separated from each other. It was however, not possible to discriminate between the different antifungal concentrations at this stage.

B. Ventilator associated pneumonia:

- The e-nose successfully discriminated the *in vitro* bacterial/yeast cultures from the controls. Although, it could not differentiate between all the individual species, it differentiated between the four basic groups (gram positive, gram negative, fungi and controls) with 83% accuracy.
- The microbiology culture results were successfully correlated with the e-nose sensor responses for the clinical samples.
- Based on the volatile fingerprints of the clinical samples, reasonably good discrimination between the four treatment groups was obtained, with 63% classification accuracy.

C. Oesophageal Cancer:

- The e-nose with hybrid sensors outperformed the e-nose housing conducting polymer sensors in terms of their discriminatory abilities in this study.
- There was clear differentiation between the controls and the various cell lines (normal oesophageal, Barrett's oesophagus, squamous cell carcinoma, colorectal carcinoma) when incubated for a short period (one to four hours). After 24 hour incubation only the squamous cell carcinoma cell line (OE21) could be distinguished from the others and controls.
- SIFT-MS was unable to detect the presence of specific markers or identifying compounds due to the headspace being poorly concentrated. However, two unidentified compounds at m/z 44 and 98 were detected in the cell lines but not in the controls and background, also in small amounts.

- No distinctive pattern was observed based on the volatile profiles from the clinical samples, where none of the upper gastrointestinal pathologies could be distinguished.

The use of qualitative volatile fingerprinting by means of electronic nose technology is a useful approach for the early discrimination of pathogenic micro-organisms as well as other non-microbial conditions. It holds promising prospects for the future where it may serve to be an economic, easily operative and rapid screening method.

## *7.2 Suggestions for future work*

The following section lists some suggestions for continuing the present research which could eventually take it a step closer to being an onsite routine clinical device.

A. Dermatophytes:

- Incorporating keratin based nutrients in the medium followed by volatile analysis. This might enhance the volatile production especially of the slower growing species and determine if addition of keratin causes a major change in volatile fingerprints.
- Another alternative would be using human skin explants as the substrate rather than agar or broth. Skin explants have been recently used to study the invasive mechanism of *T. mentagrophytes* (Kaufman *et al.*, 2007) as well as for delivery of biological macromolecules for gene therapy (Coulman *et al.*, 2006). This could be an approach in bringing the study closer to clinical scenarios that might possibly help in establishing whether the volatile profiles might differ from type cultures and be closer to clinical specimens.

- Increasing the number of samples for artificial neural network analysis, in order to obtain a robust and accurate predictive model.
- Application of the procedures to clinical samples, preferably in optimised liquid media, and validating the approach by exploiting the built predictive neural network as a classifier for unknown specimens.
- Investigating the presence of the same or new marker/identifier compounds by including the different strains of the *Trichophyton* and *Microsporum* species using the GC-MS and SIFT-MS techniques, thus further facilitating intra-strain comparisons.
- Screening a range of other current antifungal drugs, in order to correlate their volatile profile patterns and providing possible therapeutic alternatives by finding novel ones.
- Determining the changes, if any, in the volatile fingerprints based on the effect of the antifungals on the different strains of the dermatophytes.
- Monitoring drug resistance using the volatile production patterns of these pathogenic fungi.

B. Ventilator associated pneumonia:

- Use of a more specific or enriched broth medium rather than nutrient broth, to enable growth of all bacterial and yeast species/strains of the clinical isolates, which might enhance volatile production and therefore probably facilitate in differentiating the individual species.
- Attempt to mimic the clinical sample, by making the microbial suspensions in saline or Ringer's solution, in order to observe and if possible correlate any difference or similarities in the volatile profiles in both clinical and non-clinical instances. These results might eventually help in identifying whether the cause

of clinical discrimination is owing to the bacterial volatiles or due to products of the host's immune responses in the lavage fluid.

- Preparing mixed bacterial suspensions or cultures of the most important species in the laboratory to try to discriminate between them and individual species, and eventually comparing with clinical samples as certain patients were found to be infected by multiple organisms.
- Determining the effect of various antibiotics on the volatile fingerprints of the different bacteria and yeast species/strains, as most of the affected individuals are normally already on antibiotic therapy.
- Enriching the volatile production of clinical samples by either spiking with specific enzymes or adding to broth for a short time period prior to headspace analysis. Similar to the procedure adopted by Pavlou *et al.* (2002a; 2004) for diagnosing tuberculosis or urinary tract infections, but those studies dealt with a much smaller set/number of micro-organisms.
- Determining the compounds responsible for discrimination of the clinical isolates and clinical samples by means of GC-MS and SIFT-MS.
- If possible, obtaining clinical samples where the patients have not yet been administered with antibiotics and comparing their volatile profile patterns against those who have been treated with drugs.
- Application of artificial neural networks for building a predictive model for classification of unknowns.

C. Oesophageal cancer:

- Finding an alternative sampling procedure for the cell lines for headspace analysis with the hybrid sensor array.

- Using more concentrated cell cultures, i.e. by combining the cell cultures from two or three flasks that would increase the cell density which could in turn lead to higher volatile production, thus enabling better discrimination between the individual cell lines.
- Measuring the sample headspace without changing the medium when grown in the glass vials. This would possibly prevent any loss of cells, if at all, whilst changing the media.
- Use of Nalophan bags for concentrating the headspace for SIFT-MS analysis, with the culture flasks being placed in them individually and incubated in 5% $CO_2$ atmosphere. This might enable better identification of possible markers.
- Increasing the number of patient samples for analysis and trying breath analysis as an alternative to endoscopic gas traps, this could serve to be a better and non-invasive form of diagnosis.

# *REFERENCES*

## *Bibliography*

**Agatonovic-Kustrin, S. & Beresford, R. (2000).** Basic concepts of artificial neural network (ANN) modelling and its application in pharmaceutical research. *Journal of Pharmaceutical and Biomedical Analysis* **22**, 717-727.

**Al-Haddad, L., Morris, C. W. & Boddy, L. (2000).** Training radial basis function neural networks: Effects of training set size and imbalanced training sets. *Journal of Microbiological Methods* **43**, 33-44.

**Ali-Shtayeh, M. S. & Abu Ghdeib, S. I. (1999).** Antifungal activity of plant extracts against dermatophytes. *Mycoses* **42**, 665-672.

**Allardyce, R. A., Hill, A. L. & Murdoch, D. R. (2006).** The rapid evaluation of bacterial growth and antibiotic susceptibility in blood cultures by selected ion flow tube mass spectrometry. *Diagnostic Microbiology and Infectious Disease* **55**, 255-261.

**Arabatzis, M., Bruijnesteijn van Coppenraet, L. E. S., Kuijper, E. J. & other authors (2007).** Diagnosis of common dermatophyte infections by a novel multiplex real-time polymerase chain reaction detection/identification scheme. *British Journal of Dermatology* **157**, 681-689.

**Aronzon, A., Hanson, C. W. & Thaler, E. R. (2005).** Differentiation between cerebrospinal fluid and serum with electronic nose. *Otolaryngology - Head and Neck Surgery* **133**, 16-19.

**Artursson, T., Eklöv, T., Lundström, I., Mårtensson, P., Sjöström, M. & Holmberg, M. (2000).** Drift correction for gas sensors using multivariate methods. *Journal of Chemometrics* **14**, 711-723.

**Baby, R. E., Cabezas, M. & Walsoe de Reca, E. N. (2000).** Electronic nose: A useful tool for monitoring environmental contamination. *Sensors and Actuators B: Chemical* **69**, 214-218.

**Bachinger, T. & Mandenius, C.-F. (2000).** Searching for process information in the aroma of cell cultures. *Trends in Biotechnology* **18**, 494-500.

**Baeza, L. C. & Giannini, M. J. S. M. (2004).** Strain differentiation of *Trichophyton rubrum* by random amplification of polymorphic DNA (RAPD) *Revista do Instituto de Medicina Tropical de São Paulo* **46** 339-341

**Baeza, L. C., Matsumoto, M. T., Almeida, A. M. F. & Mendes-Giannini, M. J. S. (2006).** Strain differentiation of *Trichophyton rubrum* by randomly amplified polymorphic DNA and analysis of rDNA nontranscribed spacer. *J Med Microbiol* **55**, 429-436.

**Balseiro, S. C. & Correia, H. R. (2006).** Is olfactory detection of human cancer by dogs based on major histocompatibility complex-dependent odour components? - A possible cure and a precocious diagnosis of cancer. *Medical Hypotheses* **66**, 270-272.

**Basheer, I. A. & Hajmeer, M. (2000).** Artificial neural networks: Fundamentals, computing, design and application. *Journal of Microbiological Methods* **43**, 3–31.

**Bateman, A. C. (2003).** Pathology of upper gastrointestinal tumours. *Surgery (Oxford)* **21**, iii-vi.

**Baughman, R. P. (2005a).** Microbiologic diagnosis of ventilator-associated pneumonia. *Clin Chest Med* **26**, 81-86.

**Baughman, R. P. (2005b).** Diagnosis of ventilator-associated pneumonia. *Microbes and Infection* **7**, 262-267.

**Binstock, J. M. (2007).** Molecular biology techniques for identifying dermatophytes and their possible use in diagnosing onychomycosis in human toenail. *Journal of the American Podiatric Medical Association* **97**, 134-144.

**Boilot, P., Hines, E. L., Gardner, J. W., Pitt, R., John, S., Mitchell, J. & Morgan, D. W. (2002).** Classification of bacteria responsible for ENT and eye infections using the Cyranose system. *IEEE SENSORS JOURNAL* **2**, 247-253.

**Bourgeois, W. & Stuetz, R. M. (2002).** Use of a chemical sensor array for detecting pollutants in domestic wastewater. *Water Research* **36**, 4505-4512.

**Brereton, R. G. (2003).** *Chemometrics: Data analysis for the laboratory and chemical plants*. Chichester: Wiley.

**Brillowska-Dąbrowska, A., Saunte, D. M. & Arendrup, M. C. (2007).** Five-hour diagnosis of dermatophyte nail infections with specific detection of *Trichophyton rubrum*. *J Clin Microbiol* **45**, 1200-1204.

**Brudzewski, K., Osowski, S., Markiewicz, T. & Ulaczyk, J. (2006).** Classification of gasoline with supplement of bio-products by means of an electronic nose and SVM neural network. *Sensors and Actuators B: Chemical* **113**, 135-141.

**Cabañes, F. J., Sahgal, N. & Magan, N. (2006).** Identification of black *Aspergilli* species responsible for Ochratoxin A contamination of food using qualitative volatile fingerprints. In *8th International Mycological Congress* pp. 137, PS136-238. Cairns, Australia.

**Cancer Research U.K. (2007).** Cancer Stats. *http://info.cancerresearchuk.org/cancerstats (last accessed 19th November 2007).*

**Canhoto, O. & Magan, N. (2003).** Potential for detection of microorganisms and heavy metals in potable water using electronic nose technology. *Biosensors and Bioelectronics* **18**, 751-754.

**Canhoto, O., Pinzari, F., Fanelli, C. & Magan, N. (2004).** Application of electronic nose technology for the detection of fungal contamination in library paper. *International Biodeterioration & Biodegradation* **54**, 303-309.

**Canhoto, O. & Magan, N. (2005).** Electronic nose technology for the detection of microbial and chemical contamination of potable water. *Sensors and Actuators B: Chemical* **106**, 3-6.

**Carroll, W., Lenney, W., Wang, T., Španěl, P., Alcock, A. & Smith, D. (2005).** Detection of volatile compounds emitted by *Pseudomonas aeruginosa* using selected ion flow tube mass spectrometry. *Pediatric Pulmonology* **39**, 452-456.

**Casalinuovo, I. A., Di Pierro, D., Bruno, E., Di Francesco, P. & Coletta, M. (2006).** Experimental use of a new surface acoustic wave sensor for the rapid identification of bacteria and yeasts. *Letters in Applied Microbiology* **42**, 24-29.

**Cavalcanti, M., Valencia, M. & Torres, A. (2005).** Respiratory nosocomial infections in the medical intensive care unit. *Microbes and Infection* **7**, 292-301.

**Chandiok, S., Crawley, B. A., Oppenheim, B. A., Chadwick, P. R., Higgins, S. & Persaud, K. C. (1997).** Screening for bacterial vaginosis: A novel application of artificial nose technology. *J Clin Pathol* **50**, 790-791.

**Chastre, J. & Fagon, J.-Y. (2002).** Ventilator-associated pneumonia. *Am J Respir Crit Care Med* **165**, 867-903.

**Chastre, J. & Luyt, C. E. (2007).** Optimising the duration of antibiotic therapy for ventilator-associated pneumonia. *Eur Respir Rev* **16**, 40-44.

**Chen, X., Cao1, M., Li, Y., Hu1, W., Wang, P., Ying, K. & Pan, H. (2005).** A study of an electronic nose for detection of lung cancer based on a virtual SAW gas sensors array and imaging recognition method. *Measurement Science and Technology* **16**, 1535–1546.

**Cimato, A., Dello Monaco, D., Distante, C., Epifani, M., Siciliano, P., Taurino, A. M., Zuppa, M. & Sani, G. (2006).** Analysis of single-cultivar extra virgin olive oils by means of an Electronic Nose and HS-SPME/GC/MS methods. *Sensors and Actuators B: Chemical* **114**, 674-680.

**Cometto-Muñiz, J. E. (2002).** Chemical sensing in humans and machines. In *Handbook of machine olfaction*. Edited by T. C. Pearce, S. S. Schiffman, H. T. Nagle & J. W. Gardner. Weinheim: Wiley-VCH Verlag.

**Cook, D. J., Walter, S. D., Cook, R. J., Griffith, L. E., Guyatt, G. H., Leasa, D., Jaeschke, R. Z. & Brun-Buisson, C. (1998).** Incidence of and risk factors for ventilator associated pneumonia in critically ill patients. *Annals of Internal Medicine* **129**, 433-440.

**Coulman, S. A., Barrow, D., Anstey, A., Gateley, C., Morrissey, A., Wilke, N., Allender, C., Brain, K. & Birchall, J. C. (2006).** Minimally invasive cutaneous delivery of macromolecules and plasmid DNA via microneedles. *Current Drug Delivery* **3**, 65-75.

**Craven, D. E. (2000).** Epidemiology of ventilator-associated pneumonia. *Chest* **117**, 186S-187.

**Craven, M. A., Gardner, J. W. & Bartlett, P. N. (1996).** Electronic noses - development and future prospects. *TrAC Trends in Analytical Chemistry* **15**, 486-493.

**Deisingh, A. K., Stone, D. C. & Thompson, M. (2004).** Applications of electronic noses and tongues in food analysis. *International Journal of Food Science and Technology* **39**, 587-604.

**Demuth, H. & Beale, M. (2006).** Neural Network Toolbox v 5.0 for use with MATLAB v 7. *THE MATH WORKS Inc Natick, MA, USA*.

**Demyttenaere, J. C. R., Morina, R. M. & Sandra, P. (2003).** Monitoring and fast detection of mycotoxin-producing fungi based on headspace solid-phase microextraction and headspace sorptive extraction of the volatile metabolites. *Journal of Chromatography A* **985**, 127-135.

**Deng, C., Zhang, X. & Li, N. (2004).** Investigation of volatile biomarkers in lung cancer blood using solid-phase microextraction and capillary gas chromatography-mass spectrometry. *Journal of Chromatography B* **808**, 269-277.

**Di Francesco, F., Fuoco, R., Trivella, M. G. & Ceccarini, A. (2005).** Breath analysis: trends in techniques and clinical applications. *Microchemical Journal* **79**, 405-410.

**Di Natale, C., Macagnano, A., Martinelli, E., Paolesse, R., D'Arcangelo, G., Roscioni, C., Finazzi-Agro, A. & D'Amico, A. (2003).** Lung cancer identification by the analysis of breath by means of an array of non-selective gas sensors. *Biosensors and Bioelectronics* **18**, 1209-1218.

**Di Salvo, A. (2005).** Introduction to mycology. In *Microbiology and Immunology On-line,*. Edited by R. C. Hunt: http://pathmicro.med.sc.edu/mycology/mycology-1.htm University of South Carolina School of Medicine.

**Dickinson, T. A., White, J., Kauer, J. S. & Walt, D. R. (1998).** Current trends in 'artificial-nose' technology. *Trends in Biotechnology* **16**, 250-258.

**Diskin, A. M., Španěl, P. & Smith, D. (2003).** Time variation of ammonia, acetone, isoprene and ethanol in breath: a quantitative SIFT-MS study over 30 days. *Physiol Meas* **24** 107-119

**Dixon, S. J., Xu, Y., Brereton, R. G., Soini, H. A., Novotny, M. V., Oberzaucher, E., Grammer, K. & Penn, D. J. (2007).** Pattern recognition of gas chromatography mass spectrometry of human volatiles in sweat to distinguish the sex of subjects and determine potential discriminatory marker peaks. *Chemometrics and Intelligent Laboratory Systems* **87**, 161-172.

**Dutta, R., Hines, E., Gardner, J. & Boilot, P. (2002).** Bacteria classification using Cyranose 320 electronic nose. *BioMedical Engineering OnLine* **1**, 4-10.

**Dutta, R., Hines, E. L., Gardner, J. W., Kashwan, K. R. & Bhuyan, M. (2003).** Determination of tea quality by using a neural network based electronic nose. *IEEE: Neural Networks* **1**, 404-409.

**Dutta, R., Morgan, D., Baker, N., Gardner, J. W. & Hines, E. L. (2005).** Identification of *Staphylococcus aureus* infections in hospital environment: electronic nose based approach. *Sensors and Actuators B: Chemical* **109**, 355-362.

**Eisenberger, C. F., Stoecklein, N. H., Jazra, S., Hosch, S. B., Peiper, M., Scheunemann, P., Schulte am Esch, J. & Knoefel, W. T. (2006).** The detection of oesophageal adenocarcinoma by serum microsatellite analysis. *EJSO* **32**, 954-960.

**Ellis, D. (2007).** Mycology Online. http://www.mycology.adelaide.edu.au/ *(last accessed 21st September 2007)*.

**Esteban, A., Abarca, M. L. & Cabañes, F. J. (2005).** Comparison of disk diffusion method and broth microdilution method for antifungal susceptibility testing of dermatophytes. *Medical Mycology* **43**, 61-66.

**Evans, P., Persaud, K. C., McNeish, A. S., Sneath, R. W., Hobson, N. & Magan, N. (2000).** Evaluation of a radial basis function neural network for the determination of wheat quality from electronic nose data. *Sensors and Actuators B: Chemical* **69**, 348-358.

**Everitt, B. S. & Dunn, G. (2001).** *Applied multivariate data analysis*, 2nd edn. London: Arnold.

**Faggi, E., Pini, G., Campisi, E., Bertellini, C., Difonzo, E. & Mancianti, F. (2001).** Application of PCR to distinguish common species of dermatophytes. *J Clin Microbiol* **39**, 3382-3385.

**Fagon, J. Y. & Chastre, J. (2005).** Antimicrobial treatment of hospital-acquired pneumonia. *Clin Chest Med* **26**, 97-104.

**Falasconi, M., Gobbi, E., Pardo, M., Della Torre, M., Bresciani, A. & Sberveglieri, G. (2005a).** Detection of toxigenic strains of *Fusarium verticillioides* in corn by electronic olfactory system. *Sensors and Actuators B: Chemical* **108**, 250-257.

**Falasconi, M., Pardo, M., Sberveglieri, G., Battistutta, F., Piloni, M. & Zironi, R. (2005b).** Study of white truffle aging with SPME-GC-MS and the Pico2-electronic nose. *Sensors and Actuators B: Chemical* **106**, 88-94.

**Falasconi, M., Pardo, M., Sberveglieri, G., Ricco, I. & Bresciani, A. (2005c).** The novel EOS835 electronic nose and data analysis for evaluating coffee ripening. *Sensors and Actuators B: Chemical* **110**, 73-80.

**Favre, B., Hofbauer, B., Hildering, K.-S. & Ryder, N. S. (2003).** Comparison of *in vitro* activities of 17 antifungal drugs against a panel of 20 dermatophytes by using a microdilution assay. *J Clin Microbiol* **41**, 4817-4819.

**Fend, R., Bessant, C., Williams, A. J. & Woodman, A. C. (2004).** Monitoring haemodialysis using electronic nose and chemometrics. *Biosensors and Bioelectronics* **19**, 1581-1590.

**Fend, R., Geddes, R., Lesellier, S. & other authors (2005).** Use of an electronic nose to diagnose *Mycobacterium bovis* infection in badgers and cattle. *Journal of Clinical Microbiology* **43**, 1745-1751.

**Fernandez-Torres, B., Cabanes, F. J., Carrillo-Munoz, A. J., Esteban, A., Inza, I., Abarca, L. & Guarro, J. (2002).** Collaborative evaluation of optimal antifungal susceptibility testing conditions for dermatophytes. *J Clin Microbiol* **40**, 3999-4003.

**Flanders, S. A., Collard, H. R. & Saint, S. (2006).** Nosocomial pneumonia: state of the science. *Am J Infect Control* **34**, 84-93.

**Fujitani, S. & Yu, V. L. (2006).** Diagnosis of ventilator-associated pneumonia: Focus on nonbronchoscopic techniques (nonbronchoscopic bronchoalveolar lavage, including mini-BAL, blinded protected specimen brush, and blinded bronchial sampling) and endotracheal aspirates. *J Intensive Care Med* **21**, 17-21.

**Fuller, L. C., Child, F. C., Midgley, M. & Higgins, E. M. (2003).** Scalp ringworm in south east London and an analysis of a cohort of patients from a paediatric dermatology department. *British Journal of Dermatology* **148**, 985-988.

**Garcia, M., Fernandez, M. J., Fontecha, J. L., Lozano, J., Santos, J. P., Aleixandre, M., Sayago, I., Gutierrez, J. & Horrillo, M. C. (2006).** Differentiation of red wines using an electronic nose based on surface acoustic wave devices. *Talanta* **68**, 1162-1165.

**Gardner, J. W. & Bartlett, P. N. (1994).** A brief history of electronic noses. *Sensors and Actuators B: Chemical* **18**, 210-211.

**Gardner, J. W. & Bartlett, P. N. (1999).** *Electronic noses: principles and applications*. Oxford: Oxford University Press.

**Gendron, K. B., Hockstein, N. G., Thaler, E. R., Vachani, A. & Hanson, C. W. (2007).** *In vitro* discrimination of tumor cell lines with an electronic nose. *Otolaryngology - Head and Neck Surgery* **137**, 269-273.

**Ghahfarokhi, M. S., Goodarzi, M., Abyaneh, M. R., Al-Tiraihi, T. & Seyedipour, G. (2004).** Morphological evidences for onion-induced growth inhibition of *Trichophyton rubrum* and *Trichophyton mentagrophytes*. *Fitoterapia* **75**, 645-655.

**Gibson, T. D., Prosser, O., Hulbert, J. N., Marshall, R. W., Corcoran, P., Lowery, P., Ruck-Keene, E. A. & Heron, S. (1997).** Detection and simultaneous identification of microorganisms from headspace samples using an electronic nose. *Sensors and Actuators B: Chemical* **44**, 413-422.

**Goodner, K. L., Dreher, J. G. & Rouseff, R. L. (2001).** The dangers of creating false classifications due to noise in electronic nose and similar multivariate analyses. *Sensors and Actuators B: Chemical* **80**, 261-266.

**Göpel, W. (2000).** From electronic to bioelectronic olfaction, or: from artificial "moses" to real noses. *Sensors and Actuators B: Chemical* **65**, 70-72.

**Gräser, Y., Kühnisch, J. & Presber, W. (1999).** Molecular markers reveal exclusively clonal reproduction in *Trichophyton rubrum*. *J Clin Microbiol* **37**, 3713-3717.

**Gupta, A. K. & Tu, L. Q. (2006).** Dermatophytes: Diagnosis and treatment. *Journal of the American Academy of Dermatology* **54**, 1050-1055.

**Gurgel, L. A., Sidrim, J. J. C., Martins, D. T., Filho, V. C. & Rao, V. S. (2005).** *In vitro* antifungal activity of dragon's blood from *Croton urucurana* against dermatophytes. *Journal of Ethnopharmacology* **97**, 409-412.

**Halket, J. M., Przyborowska, A., Stein, S. E., Mallard, W. G., Down, S. & Chalmers, R. A. (1999).** Deconvolution gas chromatography/mass spectrometry of urinary organic acids - potential for pattern recognition and automated identification of metabolic disorders. *Rapid Communications in Mass Spectrometry* **13**, 279-284.

**Hammoud, Z. T., Badve, S., Saxena, R., Kesler, K. A., Rieger, K., Malkas, L. H. & Hickey, R. J. (2007a).** A novel biomarker for the detection of esophageal adenocarcinoma. *J Thorac Cardiovasc Surg* **133**, 82-87.

**Hammoud, Z. T., Dobrolecki, L., Kesler, K. A., Rahmani, E., Rieger, K., Malkas, L. H. & Hickey, R. J. (2007b).** Diagnosis of esophageal adenocarcinoma by serum proteomic pattern. *Ann Thorac Surg* **84**, 384-392.

**Hanson, C. W. & Thaler, E. R. (2005).** Electronic nose prediction of a clinical pneumonia score: biosensors and microbes. *Anesthesiology* **102**, 63-68.

**Haugen, J.-E., Tomic, O. & Kvaal, K. (2000).** A calibration method for handling the temporal drift of solid state gas-sensors. *Analytica Chimica Acta* **407**, 23-39.

**Hay, R. J. (2003).** Cutaneous and subcutaneous mycoses. In *Clinical Mycology*, pp. 456-473. Edited by E. J. Anaissie, M. R. McGinnis & M. A. Pfaller. Philadelphia, USA: Churchill Livingstone.

**Hines, E. L., Boilot, P., Gardner, J. W. & Gongora, M. A. (2002).** Pattern analysis for electronic noses. In *Handbook of machine olfaction*. Edited by T. C. Pearce, S. S. Schiffman, H. T. Nagle & J. W. Gardner. Weinheim: Wiley-VCH Verlag.

**Hiruma, M. & Yamaguchi, H. (2003).** Dermatophytes. In *Clinical Mycology*, pp. 370-379. Edited by E. J. Anaissie, M. R. McGinnis & M. A. Pfaller. Philadelphia, USA: Churchill Livingstone.

**Hollemeyer, K., Jager, S., Altmeyer, W. & Heinzle, E. (2005).** Proteolytic peptide patterns as indicators for fungal infections and nonfungal affections of human nails

measured by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Analytical Biochemistry* **338**, 326-331.

**Holmes, R. S. & Vaughan, T. L. (2007).** Epidemiology and pathogenesis of esophageal cancer. *Seminars in Radiation Oncology* **17**, 2-9.

**Hope, R., Jestoi, M. & Magan, N. (2002).** Multitarget environmental approach for control of growth and toxin production by *Fusarium culmorum* using essential oils and antioxidants. In *Advances in Stored Products Protection Proceedings of the Eighth International Working Conference on Stored Product Protection, 22–26 July, York, UK*. Edited by P. F. Credland, M. Armitage, C. H. Bell, P. M. Cogan & E. Highley. Wallingford: CABI International.

**Hu, N., Roth, M. J., Polymeropolous, M. & other authors (2000).** Identification of novel regions of allelic loss from a genomewide scan of esophageal squamous-cell carcinoma in a high-risk Chinese population. *Genes, Chromosomes and Cancer* **27**, 217-228.

**Hunter, J. D. (2006).** Ventilator associated pneumonia. *Postgrad Med J* **82**, 172-178.

**Jackson, C. J., Barton, R. C. & Evans, E. G. V. (1999).** Species identification and strain differentiation of dermatophyte fungi by analysis of ribosomal-DNA intergenic spacer regions. *Journal of Clinical Microbiology* **37**, 931-936.

**Jackson, C. J., Barton, R. C., Kelly, S. L. & Evans, E. G. V. (2000).** Strain identification of *Trichophyton rubrum* by specific amplification of subrepeat elements in the ribosomal DNA nontranscribed spacer. *J Clin Microbiol* **38**, 4527-4534.

**Jelén, H. H. & Grabarkiewicz-Szczesna, J. (2005).** Volatile compounds of *Aspergillus* strains with different abilities to produce Ochratoxin A. *J Agric Food Chem* **53**, 1678-1683.

**Jemal, A., Siegel, R., Ward, E., Murray, T., Xu, J. & Thun, M. J. (2007).** Cancer Statistics, 2007. *CA Cancer J Clin* **57**, 43-66.

**Jurs, P. C., Bakken, G. A. & McClelland, H. E. (2000).** Computational methods for the analysis of chemical sensor array data from volatile analytes. *Chem Rev* **100**, 2649-2678.

**Kac, G., Bougnoux, M. E., Feuilhade de Chauvin, M., Sene, S. & Derouin, F. (1999).** Genetic diversity among *Trichophyton mentagrophytes* isolates using random amplified polymorphic DNA method. *British Journal of Dermatology* **140**, 839-844.

**Kamiya, A., Kikuchi, A., Tomita, Y. & Kanbe, T. (2004).** PCR and PCR-RFLP techniques targeting the DNA topoisomerase II gene for rapid clinical diagnosis of the etiologic agent of dermatophytosis. *Journal of Dermatological Science* **34**, 35-48.

**Kardjeva, V., Summerbell, R., Kantardjiev, T., Devliotou-Panagiotidou, D., Sotiriou, E. & Graser, Y. (2006).** Forty-eight-hour diagnosis of onychomycosis with subtyping of *Trichophyton rubrum* strains. *J Clin Microbiol* **44**, 1419-1427.

**Karlshøj, K., Nielsen, P. V. & Larsen, T. O. (2007).** Differentiation of closely related fungi by electronic nose analysis. *Journal of Food Science* **72**, M187-M192.

**Kato, S., Burke, P. K., Fenick, D. J., Taatjes, D. J., Bierbaum, V. M. & Koch, T. H. (2000).** Mass spectrometric measurement of the formaldehyde generated in breast cancer cells upon treatment with anthracylin antitumor drugs. *Chem Res Toxicol* **13**, 509-516.

**Kato, S., Burke, P. K., Koch, T. H. & Bierbaum, V. M. (2001).** Formaldehyde in human cancer cells: Detection by preconcentration-chemical ionisation mass spectrometry. *Anal Chem* **73**, 2992-2997.

**Kaufman, G., Horwitz, B. A., Duek, L., Ullman, Y. & Berdicevsky, I. (2007).** Infection stages of the dermatophyte pathogen *Trichophyton*: Microscopic characterization and proteolytic enzymes. *Medical Mycology* **45**, 149 - 155.

**Kern, M. E. & Blevins, K. S. (1997).** *Medical Mycology: A self-instructional text*, 2nd edn. Philadelphia, USA: F.A. Davis.

**Keshri, G., Magan, N. & Voysey, P. (1998).** Use of an electronic nose for the early detection and differentiation between spoilage fungi. *Letters in Applied Microbiology* **27**, 261-264.

**Keshri, G. & Magan, N. (2000).** Detection and differentiation between mycotoxigenic and non-mycotoxigenic strains of two *Fusaria* spp. using volatile production profiles and hydrolytic enzymes. *Journal of Applied Microbiology* **89**, 825-833.

**Keshri, G., Voysey, P. & Magan, N. (2002).** Early detection of spoilage moulds in bread using volatile production patterns and quantitative enzyme assays. *Journal of Applied Microbiology* **92**, 165-172.

**Kim, J. A., Takahashi, Y., Tanaka, R., Fukushima, K., Nishimura, K. & Miyaji, M. (2000).** Identification and subtyping of *Trichophyton mentagrophytes* by random amplified polymorphic DNA. *Mycoses* **44**, 157-165.

**Kirtland, S. H., Corley, D. E., Winterbauer, R. H., Springmeyer, S. C., Casey, K. R., Hampson, N. B. & Dreis, D. F. (1997).** The diagnosis of ventilator-associated pneumonia: A comparison of histologic, microbiologic, and clinical criteria. *Chest* **112**, 445-457.

**Koc, A. N., Silici, S., Ayangil, D., Ferahbas, A. & Cankaya, S. (2005).** Comparison of *in vitro* activities of antifungal drugs and ethanolic extract of propolis against *Trichophyton rubrum* and *T. mentagrophytes* by using a microdilution assay. *Mycoses* **48**, 205-210.

**Koeman, M., van der Ven, A. J. A. M., Ramsay, G., Hoepelman, I. M. & Bonten, M. J. M. (2001).** Ventilator-associated pneumonia: Recent issues on pathogenesis, prevention and diagnosis. *Journal of Hospital Infection* **49**, 155-162.

**Koenig, S. M. & Truwit, J. D. (2006).** Ventilator-associated pneumonia: Diagnosis, treatment, and prevention. *Clinical Microbiology Reviews* **19**, 637–657.

**Kollef, M. H. (2003).** The importance of appropriate initial antibiotic therapy for hospital-acquired infections. *The American Journal of Medicine* **115**, 582-584.

**Kollef, M. H. (2005).** What is ventilator associated pneumonia and why is it important? *Respiratory Care* **50**, 714-724.

**Kreij, K., Mandenius, C.-F., Clemente, J. & other authors (2005).** On-line detection of microbial contaminations in animal cell reactor cultures using an electronic nose device. *Cytotechnology* **48**, 41-58.

**Kumbasar, B. (2002).** Carcinoma of esophagus: radiologic diagnosis and staging. *European Journal of Radiology* **42**, 170-180.

**Kushalappa, A. C., Lui, L. H., Chen, C. R. & Lee, B. (2002).** Volatile fingerprinting (SPME-GC-FID) to detect and discriminate diseases of potato tubers. *Plant Disease* **86**, 131-137.

**Kuske, M., Rubio, R., Romain, A. C., Nicolas, J. & Marco, S. (2005).** Fuzzy k-NN applied to moulds detection. *Sensors and Actuators B: Chemical* **106**, 52-60.

**Labreche, S., Bazzo, S., Cade, S. & Chanie, E. (2005).** Shelf life determination by electronic nose: application to milk. *Sensors and Actuators B: Chemical* **106**, 199-206.

**Lamb, P. J. & Griffin, S. M. (2003).** Carcinoma of the oesophagus. *Surgery (Oxford)* **21**, 1-4.

**Lee, J. S., Yang, W. K., Han, E. Y., Lee, S. Y., Park, Y. H., Lim, M. A., Chung, H. S. & Park, J. H. (2007).** Monitoring precursor chemicals of methamphetamine through enantiomer profiling. *Forensic Science International* **173**, 68-72.

**Lightdale, C. J. (1999).** Esophageal cancer. *The American Journal of Gastroenterology* **94**, 20-29.

**Liu, D., Coloe, S., Pedersen, J. & Baird, R. (1996).** Use of arbitrarily primed polymerase chain reaction to differentiate *Trichophyton* dermatophytes. *FEMS Microbiology Letters* **136**, 147-150.

**Liu, D., Coloe, S., Baird, R. & Pedersen, J. (1997).** Rapid differentiation of *Microsporum* dermatophytes based on arbitrarily primed PCR amplification. *Opportunistic Pathogens* **9**, 3-6.

**Liu, D., Coloe, S., Baird, R. & Pedersen, J. (2000).** Application of PCR to the identification of dermatophyte fungi. *J Med Microbiol* **49**, 493-497.

**Lozano, J., Santos, J. P. & Horrillo, M. C. (2005).** Classification of white wine aromas with an electronic nose. *Talanta* **67**, 610-616.

**Lu, N., Hu, N., Li, W.-J., Roth, M. J., Wang, C., Su, H., Wang, Q.-H., Taylor, P. R. & Dawsey, S. M. (2003).** Microsatellite alterations in esophageal dysplasia and squamous cell carcinoma from laser capture microdissected endoscopic biopsies. *Cancer Letters* **189**, 137-145.

**Machouart-Dubach, M., Lacroix, C., de Chauvin, M. F., Le Gall, I., Giudicelli, C., Lorenzo, F. & Derouin, F. (2001).** Rapid discrimination among dermatophytes, *Scytalidium* spp., and other fungi with a PCR-restriction fragment length polymorphism ribotyping method. *Journal of Clinical Microbiology* **39**, 685-690.

**Magan, N. & Evans, P. (2000).** Volatiles as an indicator of fungal activity and differentiation between species, and the potential use of electronic nose technology for early detection of grain spoilage. *Journal of Stored Products Research* **36**, 319-340.

**Magan, N., Pavlou, A. & Chrysanthakis, I. (2001).** Milk-sense: a volatile sensing system recognises spoilage bacteria and yeasts in milk. *Sensors and Actuators B: Chemical* **72**, 28-34.

**Magan, N. & Sahgal, N. (2007).** Electronic nose for quality and safety control. In *Advances in Food Diagnostics*, pp. 119-129. Edited by L. Nollet & F. Toldra: Blackwell Publishing.

**Marín, S., Vinaixa, M., Brezmes, J., Llobet, E., Vilanova, X., Correig, X., Ramos, A. J. & Sanchis, V. (2007).** Use of a MS-electronic nose for prediction of early fungal spoilage of bakery products. *International Journal of Food Microbiology* **114**, 10-16.

**Marsili, R. T. (1999).** SPME-MS-MVA as an electronic nose for the study of off-flavors in milk. *J Agric Food Chem* **47**, 648-654.

**McEachern, R. & Campbell, G. D. (1998).** Hospital acquired pneumonia: epidemiology, etiology and treatment. *Infect Dis Clin North Am* **12**, 761-779.

**McGruder, B. M., Atha, D. H., Wang, W. & other authors (2006).** Real-time telomerase assay of less-invasively collected esophageal cell samples. *Cancer Letters* **244**, 91-100.

**Merck Manual (1999).** Gastrointestinal disorders. In *The Merck manual of diagnosis and therapy (http://www.merck.com/mmpe/index.html)*. Edited by M. H. Beers & R. Berkow. Whitehouse Station, N.J.: Merck Research Laboratories

**Metintas, S., Kiraz, N., Arslantas, D., Akgun, Y., Kalyoncu, C., Kiremitçi, A. & Unsal, A. (2004).** Frequency and risk factors of dermatophytosis in students living in rural areas in Eskisehir, Turkey. *Mycopathologia* **157**, 379-382.

**Moens, M., Smet, A., Naudts, B., Verhoeven, J., Ieven, M., Jorens, P., Geise, H. J. & Blockhuys, F. (2006).** Fast identification of ten clinically important micro-organisms using an electronic nose. *Letters in Applied Microbiology* **42**, 121-126.

**Montag, S., Frank, M., Ulmer, H., Wernet, D., Göpel, W. & Rammensee, H. G. (2001).** "Electronic nose" detects major histocompatibility complex-dependent prerenal and postrenal odor components. *Proc Natl Acad Sci U S A* **98**, 9249-9254.

**Morehead, R. S. & Pinto, S. J. (2000).** Ventilator-associated pneumonia. *Arch Intern Med* **160**, 1926-1936.

**Morvan, M., Talou, T., Gaset, A. & Beziau, J. F. (2000).** Electronic-nose systems for control quality applications in automotive industry. *Sensors and Actuators B: Chemical* **69**, 384-388.

**Nagle, H. T., Gutierrez-Osuna, R. & Schiffman, S. S. (1998).** The how and why of electronic noses. *Spectrum, IEEE* **35**, 22-31.

**Nakamota, T. (2002).** Odor handling and delivery systems. In *Handbook of machine olfaction*. Edited by T. C. Pearce, S. S. Schiffman, H. T. Nagle & J. W. Gardner. Weinheim: Wiley-VCH Verlag.

**Nake, A., Dubreuil, B., Raynaud, C. & Talou, T. (2005).** Outdoor in situ monitoring of volatile emissions from wastewater treatment plants with two portable technologies of electronic noses. *Sensors and Actuators B: Chemical* **106**, 36-39.

**Nanto, H. & Stetter, J. R. (2002).** Introduction to chemosensors. In *Handbook of machine olfaction*. Edited by T. C. Pearce, S. S. Schiffman, H. T. Nagle & J. W. Gardner. Weinheim: Wiley-VCH Verlag.

**Needham, R. & Magan, N. (2003).** Detection and differentiation of toxigenic and non-toxigenic *Penicillium verrucosum* strains on bakery products using an electronic nose. *Aspects of Applied Biology* **68**, 217-222.

**Needham, R. (2004).** Early detection and differentiation of microbial spoilage of bread using electronic nose technology. *PhD Thesis.* Silsoe: Cranfield University.

**Needham, R., Williams, J., Beales, N., Voysey, P. & Magan, N. (2005).** Early detection and differentiation of spoilage of bakery products. *Sensors and Actuators B: Chemical* **106**, 20-23.

**Negnevitsky, M. (2002).** *Artificial Intelligence: A guide to intelligent systems*. Harlow Addison-Wesley.

**Nilsson, T., Larsen, T. O., Montanarella, L. & Madsen, J. Ø. (1996).** Application of head-space solid-phase microextraction for the analysis of volatile metabolites emitted by Penicillium species. *Journal of Microbiological Methods* **25**, 245-255.

**Nishimori, T., Tomonaga, T., Matsushita, K. & other authors (2006).** Proteomic analysis of primary esophageal squamous cell carcinoma reveals down regulation of a cell adhesion protein, periplakin. *Proteomics* **6**, 1011-1018.

**Okabayashi, K., Kano, R., Nakamura, Y., Ooka, S., Kashima, M., Mizoguchi, M., Watanabe, S. & Hasegawa, A. (1999).** Molecular confirmation of a *Trichophyton violaceum* isolate from human black-dot ringworm. **146**, 127-130.

**Otto, M. (1999).** *Chemometrics : Statistics and computer application in analytical chemistry*. Weinheim: Wiley-VCH.

**Panagou, E. Z., Kodogiannis, V. & Nychas, G. J. E. (2007).** Modelling fungal growth using radial basis function neural networks: The case of the ascomycetous fungus *Monascus ruber* van Tieghem. *International Journal of Food Microbiology* **117**, 276-286.

**Pavlou, A. K., Magan, N., Sharp, D., Brown, J., Barr, H. & Turner, A. P. F. (2000).** An intelligent rapid odour recognition model in discrimination of *Helicobacter pylori* and other gastroesophageal isolates *in vitro*. *Biosensors and Bioelectronics* **15**, 333-342.

**Pavlou, A. K., Magan, N., McNulty, C., Jones, J. M., Sharp, D., Brown, J. & Turner, A. P. F. (2002a).** Use of an electronic nose system for diagnoses of urinary tract infections. *Biosensors and Bioelectronics* **17**, 893-899.

**Pavlou, A. K., Turner, A. P. F. & Magan, N. (2002b).** Recognition of anaerobic bacterial isolates *in vitro* using electronic nose technology. *Letters in Applied Microbiology* **35**, 366-369.

**Pavlou, A. K., Magan, N., Jones, J. M., Brown, J., Klatser, P. & Turner, A. P. F. (2004).** Detection of *Mycobacterium tuberculosis* (TB) *in vitro* and *in situ* using an electronic nose in combination with a neural network system. *Biosensors and Bioelectronics* **20**, 538-544.

**Pearce, T. C. (1997).** Computational parallels between the biological olfactory pathway and its analogue 'The Electronic Nose': Part II. Sensor-based machine olfaction. *Biosystems* **41**, 69-90.

**Pearce, T. C., Schiffman, S. S., Nagle, H. T. & Gardner, J. W. (2002).** *Handbook of Machine Olfaction*. Weinheim: Wiley-VCH Verlag.

**Porzecanski, I. & Bowton, D. L. (2006).** Diagnosis and treatment of ventilator-associated pneumonia. *Chest* **130**, 597-604.

**Pugin, J. (2002).** Clinical signs and scores for the diagnosis of ventilator-associated pneumonia. *Minerva Anestesiol* **68**, 261-265.

**Pyun, M. S. & Shin, S. (2006).** Antifungal effects of the volatile oils from *Allium* plants against *Trichophyton* species and synergism of the oils with ketoconazole. *Phytomedicine* **13**, 394-400.

**Rajamäki, T., Alakomi, H.-L., Ritvanen, T., Skyttä, E., Smolander, M. & Ahvenainen, R. (2006).** Application of an electronic nose for quality assessment of modified atmosphere packaged poultry meat. *Food Control* **17**, 5-13.

**Ralhan, R., Arora, S., Chattopadhyay, T. K., Shukla, N. K. & Mathur, M. (2000).** Circulating p53 antibodies, p53 gene mutational profile and product accumulation in esophageal squamous-cell carcinoma in India. *International Journal of Cancer* **85**, 791-795.

**Reid, L. M., O'Donnell, C. P. & Downey, G. (2004).** Potential of SPME-GC and chemometrics to detect adulteration of soft fruit purees. *J Agric Food Chem* **52**, 421-427.

**Rello, J., Ollendorf, D. A., Oster, G., Vera-Llonch, M., Bellm, L., Redman, R. & Kollef, M. H. (2002).** Epidemiology and outcomes of ventilator-associated pneumonia in a large US database. *Chest* **122**, 2115-2121.

**Rinaldi, M. R. (2000).** Dermatophytosis: Epidemiological and microbiological update. *Journal of the American Academy of Dermatology* **43**, S120-124.

**Rumbak, M. J. (2002).** The pathogenesis of ventilator-associated pneumonia. *Semin Respir Crit Care Med* **23**, 427-434

**Rumbak, M. J. (2005).** Pneumonia in patients who require prolonged mechanical ventilation. *Microbes and Infection* **7**, 275-278.

**Sahgal, N., Needham, R., Cabañes, F. J. & Magan, N. (2007).** Potential for detection and discrimination between mycotoxigenic and non-toxigenic spoilage moulds using volatile production patterns: A review. *Food Additives & Contaminants* **24**, 1161-1168.

**Saini, S., Barr, H. & Bessant, C. (2001).** Sniffing out disease using the artificial nose. *Biologist* **48**, 229-233.

**Santos, D. A., Barros, M. E. S. & Hamdan, J. S. (2006).** Establishing a method of inoculum preparation for susceptibility testing of *Trichophyton rubrum* and *Trichophyton mentagrophytes*. *J Clin Microbiol* **44**, 98-101.

**Schaller, E., Zenhäusern, S., Zesiger, T., Bosset, J. O. & Escher, F. (2000).** Use of preconcentration techniques applied to a MS-based "Electronic Nose". *Analusis* **28**, 743-749.

**Schöller, C., Molin, S. & Wilkins, K. (1997).** Volatile metabolites from some gram-negative bacteria. *Chemosphere* **35**, 1487-1495.

**Scotter, J. M., Langford, V. S., Wilson, P. F., McEwan, M. J. & Chambers, S. T. (2005).** Real-time detection of common microbial volatile organic compounds from medically important fungi by Selected Ion Flow Tube-Mass Spectrometry (SIFT-MS). *Journal of Microbiological Methods* **63**, 127-134.

**Serneels, S., Moens, M., Van Espen, P. J. & Blockhuys, F. (2004).** Identification of micro-organisms by dint of the electronic nose and trilinear partial least squares regression. *Analytica Chimica Acta* **516**, 1-5.

**Shimada, H., Takeda, A., Arima, M. & other authors (2000).** Serum p53 antibody is a useful tumor marker in superficial esophageal squamous cell carcinoma. *Cancer* **89**, 1677-1683.

**Shin, J.-H., Sung, J.-H., Park, S.-J., Kim, J. A., Lee, J.-H., Lee, D.-Y., Lee, E.-S. & Yang, J.-M. (2003).** Species identification and strain differentiation of dermatophyte fungi using polymerase chain reaction amplification and restriction enzyme analysis. *Journal of the American Academy of Dermatology* **48**, 857-865.

**Shin, S. & Lim, S. (2004).** Antifungal effects of herbal essential oils alone and in combination with ketoconazole against *Trichophyton* spp. *Journal of Applied Microbiology* **97**, 1289-1296.

**Shorr, A. F. & Kollef, M. H. (2005).** Ventilator-associated pneumonia: insights from recent clinical trials. *Chest* **128**, 583-591.

**Silva, M. R. R., Oliveira, J. G., Fernandes, O. F. L., Passos, X. S., Costa, C. R., Souza, L. K. H., Lemos, J. A. & Paula, J. R. (2005).** Antifungal activity of *Ocimum gratissimum* towards dermatophytes. *Mycoses* **48**, 172-175.

**Simpanya, M. F. (2000).** Dermatophytes: Their taxonomy, ecology and pathogenicity. In *Biology of dermatophytes and other keratinophilic fungi*, pp. 1-12. Edited by R. K. S. Kushwaha & J. Guarro. Bilbao, Berekintza, 2000: Revista Iberoamericana de Micología.

**Siripatrawan, U., Linz, J. E. & Harte, B. R. (2006).** Electronic sensor array coupled with artificial neural network for detection of *Salmonella typhimurium*. *Sensors and Actuators B: Chemical* **119**, 64-69.

**Smith, D., Wang, T., Sulé-Suso, J., Španěl, P. & Haj, A. (2003).** Quantification of acetaldehyde released by lung cancer cells *in vitro* using selected ion flow tube mass spectrometry. *Rapid Communications in Mass Spectrometry* **17**, 845-850.

**Smith, D. & Španěl, P. (2005).** Selected ion flow tube mass spectrometry (SIFT-MS) for on-line trace gas analysis. *Mass Spectrometry Reviews* **24**, 661-700.

**Solomkin, J. S. (2005).** Ventilator-associated pulmonary infection: the germ theory of disease remains viable. *Microbes and Infection* **7**, 279-291.

**Španěl, P. & Smith, D. (1999).** Selected ion flow tube - mass spectrometry: detection and real-time monitoring of flavours released by food products. *Rapid Communications in Mass Spectrometry* **13**, 585-596.

**Španěl, P., Smith, D., Holland, T. A., Singary, W. A. & Elder, J. B. (1999).** Analysis of formaldehyde in the headspace of urine from bladder and prostate cancer patients using selected ion flow tube mass spectrometry. *Rapid Communications in Mass Spectrometry* **13**, 1354-1359.

**Stuetz, R. M., Fenner, R. A. & Engin, G. (1999).** Assessment of odours from sewage treatment works by an electronic nose, $H_2S$ analysis and olfactometry. *Water Research* **33**, 453-461.

**Sun, Y., Peng, Y., Chen, Y. & Shukla, A. J. (2003).** Application of artificial neural networks in the design of controlled release drug delivery systems. *Advanced Drug Delivery Reviews* **55**, 1201-1215.

**Suslick, K. S. & Rakow, N. A. (2001).** A colorimetric nose: 'Smell-Seeing'. In *Artificial Chemical Sensing: Olfaction and the Electronic Nose*, pp. 8-14. Edited by J. R. Stetter & W. R. Pensrose. Pennington, NJ: Electrochem. Soc.

**Tachimori, Y. & Kato, H. (1998).** Diagnosis and surgery of esophageal cancer. *Critical Reviews in Oncology/Hematology* **28**, 57-71.

**Thaler, E. R. & Hanson, C. W. (2006).** Use of an electronic nose to diagnose bacterial sinusitis. *American Journal of Rhinology* **20**, 170-172.

**Trihaas, J. & Nielsen, P. V. (2005).** Electronic nose technology in quality assessment: Monitoring the ripening process of Danish Blue cheese. *Journal of Food Science* **70**, E44-E49.

**Trihaas, J., Vognsen, L. & Nielsen, P. V. (2005).** Electronic nose: New tool in modelling the ripening of Danish blue cheese. *International Dairy Journal* **15**, 679-691.

**Turin, L., Riva, F., Galbiati, G. & Cainelli, T. (2000).** Fast, simple and highly sensitive double-rounded PCR assay to detect medically relevant fungi in dermatological specimens. *European Journal of Clinical Investigation* **30**, 511-518.

**Turner, A. P. F. & Magan, N. (2004).** Electronic noses and disease diagnostics. *Nature Reviews: Microbiology* **2**, 1-6.

**Turner, C., Španěl, P. & Smith, D. (2006).** A longitudinal study of ethanol and acetaldehyde in the exhaled breath of healthy volunteers using selected-ion flow-tube mass spectrometry. *Rapid Communications in Mass Spectrometry* **20**, 61-68.

**Verscheure, M., Gofflot, S., Beguin, H., Marlier, M., Belot, J. L., Norland, N. & Logany, G. (2002).** Evaluation of volatile metabolites as a taxonomic tool for identification of dermatophytes. *Mycoses* **45**, 67.

**Wady, L., Bunte, A., Pehrson, C. & Larsson, L. (2003).** Use of gas chromatography-mass spectrometry/solid phase microextraction for the identification of MVOCs from moldy building materials. *Journal of Microbiological Methods* **52**, 325-332.

**Wang, L.-D., Hong, J.-Y., Qiu, S.-L., Gao, H. & Yang, C. S. (1993).** Accumulation of p53 protein in human esophageal precancerous lesions: a possible early biomarker for carcinogenesis. *Cancer Research* **53**, 1783-1787.

**Wang, P., Tan, Y., Xie, H. & Shen, F. (1997).** A novel method for diabetes diagnosis based on electronic nose. *Biosensors and Bioelectronics* **12**, 1031-1036.

**Wang, T., Smith, D. & Španěl, P. (2004).** Selected ion flow tube, SIFT, studies of the reactions of $H_3O^+$, $NO^+$ and $O_2^+$ with compounds released by *Pseudomonas* and related bacteria. *International Journal of Mass Spectrometry* **233**, 245-251.

**Web-resource (2007).** http://www2.nose-network.org/webtutorial. *NOSE II NETWORK (last accessed 30th August 2007)*.

**Weitzman, I. & Summerbell, R. (1995).** The dermatophytes. *Clin Microbiol Rev* **8**, 240-259.

**Wilkins, M. F., Boddy, L., Morris, C. W. & Jonker, R. R. (1999).** Identification of phytoplankton from flow cytometry data by using radial basis function neural networks. *Appl Environ Microbiol* **65**, 4404-4410.

**Wilson, A. D., Lester, D. G. & Oberle, C. S. (2005).** Application of conductive polymer analysis for wood and woody plant identifications. *Forest Ecology and Management* **209**, 207-224.

**Yates, J. W. T., Chappell, M. J., Gardner, J. W., Dow, C. S., Dowson, C., Hamood, A., Bolt, F. & Beeby, L. (2005).** Data reduction in headspace analysis of blood and urine samples for robust bacterial identification. *Computer Methods and Programs in Biomedicine* **79**, 259-271.

**Zampolli, S., Elmi, I., Ahmed, F., Passini, M., Cardinali, G. C., Nicoletti, S. & Dori, L. (2004).** An electronic nose based on solid state sensor arrays for low-cost indoor air quality monitoring applications. *Sensors and Actuators B: Chemical* **101**, 39-46.

**Zhang, Z.-M., Cai, J.-J., Ruan, G.-H. & Li, G.-K. (2005).** The study of fingerprint characteristics of the emanations from human arm skin using the original sampling system by SPME-GC/MS. *Journal of Chromatography B* **822**, 244-252.

**Zhong, Z., Li, R., Li, D. & Wang, D. (1997).** Typing of common dermatophytes by random amplification of polymorphic DNA. *Jpn J Med Mycol* **38**, 239–246.

**Zhou, G., Li, H., DeCamp, D. & other authors (2002).** 2D Differential in-gel electrophoresis for the identification of esophageal scans cell cancer-specific protein markers. *Mol Cell Proteomics* **1**, 117-123.

**Zhu, L., Seburg, R. A., Tsai, E., Puech, S. & Mifsud, J.-C. (2004).** Flavor analysis in a pharmaceutical oral solution formulation using an electronic-nose. *Journal of Pharmaceutical and Biomedical Analysis* **34**, 453-461.

**Zlatkis, A., Brazell, R. & Poole, C. (1981).** The role of organic volatile profiles in clinical diagnosis. *Clin Chem* **27**, 789-797.

**Zupančič Božič, D., Vrečer, F. & Kozjek, F. (1997).** Optimization of diclofenac sodium dissolution from sustained release formulations using an artificial neural network. *European Journal of Pharmaceutical Sciences* **5**, 163-169.

**Zuppa, M., Distante, C., Persaud, K. C. & Siciliano, P. (2007).** Recovery of drifting sensor responses by means of DWT analysis. *Sensors and Actuators B: Chemical* **120**, 411-416.

# *APPENDICES*

# *Appendix A – Details on micro-organisms and human cell lines*

## A.1 Fungal Isolates

***a) Trichophyton mentagrophytes (stat. conid. of Arthroderma vanbreuseghemii)***[§§]

COLLECTION: CNCPF - National Collection of Pathogenic Fungi
STRAIN NUMBER: 224
ORGANISM_TYPE: Fungal dermatophyte
SOURCE: Human
SITE_ORIGIN: beard hairs
COUNTRY_ORIGIN: UK
YEAR_OF_RECEIPT: 1954
SPECIFIC_REMARKS: "Plus" strain. = IMI 98299.
*Dr R. E. Bowers, Gloucester*[***]

***b) Trichophyton rubrum***[§§]

COLLECTION: CNCPF - National Collection of Pathogenic Fungi
STRAIN NUMBER: 115
ORGANISM_TYPE: Fungal dermatophyte
SOURCE: Human
*India - Connaught Military H., Woking, Surrey*[***]

***c) Trichophyton verrucosum***[§§]

COLLECTION: CNCPF - National Collection of Pathogenic Fungi
STRAIN NUMBER: 685
ORGANISM_TYPE: Fungal dermatophyte
SOURCE: Human
COUNTRY_ORIGIN: UK
YEAR_OF_RECEIPT: 1986
*Mr. J. Evans, Belfast, 1986 as D 382*[***]

***d) Trichophyton violaceum***[§§]

COLLECTION: CNCPF - National Collection of Pathogenic Fungi
STRAIN NUMBER: 677
ORGANISM_TYPE: Fungal dermatophyte
SOURCE: Human
SITE_ORIGIN: nail
COUNTRY_ORIGIN: UK
YEAR_OF_RECEIPT: 1986
*Dr P. O'Neill, Lewisham H., London*[***]

[§§] http://www.ukncc.co.uk/html/Databases/search.asp
[***] http://www.hpa.org.uk/srmd/div_cdmssd_nctc/searcher.html

***e) Trichophyton rubrum D12***

Strain D12 was kindly supplied by the University of Oxford.

***f) T. rubrum - R55, R57 & R59 and T. mentagrophytes – M61, M62, M63 & M64***

The following strains R55, R57, R59, M61, M62, M63 and M64 were kindly supplied by Prof. F. J. Cabañes, Autonomous University of Barcelona, Catalonia, Spain.

## A.2 Bacterial Isolates

COLLECTION: Microbiology Department, Cheltenham General Hospital
SOURCE: Clinical isolates from patients
SITE_ORIGIN: Lung, BAL fluid aspirate

***a) 16129695[†††] - Staphylococcus species***
***Candida species***
***Klebsiella pneumoniae***

***b) 16191218 - Candida albicans***
***Enterobacter species***

***c) 15949060 - Proteus mirabilis***
***Streptococcus oralis***

***d) 16366778 - MRSA***

***e) 15943723 - Pseudomonas species***

***f) 15945707 - Candida species***

***g) 16191211 - Enterobacter cloacae***

***h) 16124960 - Klebsiella pneumoniae***

***i) 15622851 - Staphylococcus species***

***j) 15636121 - Staphylococcus species***

***k) 1562286X - Streptococcus species***

***l) 16129605 - Aerococcus species***

[†††] Hospital's microbiology laboratory identification number

## A.3 Cell Lines

### *a) HET 1A*

COLLECTION: ATCC- American Type Culture Collection[‡‡‡]
ATCC NUMBER: CRL-2692
ORGANISM: Human
SOURCE: Oesophagus
DESCRIPITION: Oesophageal epithelium (normal)
ORIGIN: Oesophageal autopsy of 25 year old black male

### *b) OE21*

COLLECTION: ECACC - European Collection of Cell Culture[§§§]
ECACC NUMBER: 96062201
ORGANISM: Human
SOURCE: Oesophagus
DESCRIPTION: Oesophageal squamous cell carcinoma
ORIGIN: Squamous carcinoma of mid oesophagus of a 74 year old Caucasian male

### *c) OE33*

COLLECTION: ECACC - European Collection of Cell Culture
ECACC NUMBER: 96070808
ORGANISM: Human
SOURCE: Oesophagus
DESCRIPTION: Oesophageal carcinoma from Barrett's metaplasia
ORIGIN: Adenocarcinoma of the lower oesophagus of a 73 year old Caucasian female

### *d) CaCO2*

COLLECTION: ATCC - American Type Culture Collection
ATCC NUMBER: HTB-37
ORGANISM: Human
SOURCE: Colon
DESCRIPITION: Colorectal adenocarcinoma
ORIGIN: Adenocarcinoma from a 72 year old Caucasian male

[‡‡‡] http://www.lgcpromochem-atcc.com/common/catalog/cellBiology/cellBiologyIndex.cfm
[§§§] http://www.ecacc.org.uk/

# *Appendix B – NST operating principle and sensor tests*

## B.1 NST operating principle

The NST 3320 Lab emission analyser consists of an automated set-up. It has an 'in' needle, through which the sample gas passes and simultaneously replaced by the filtered air (by means of a hydrocarbon filter and a silica drying column) passively as depicted in Figure B.1 by an 'out' needle. Figure B.2 illustrates the typical sensor response of raw data.

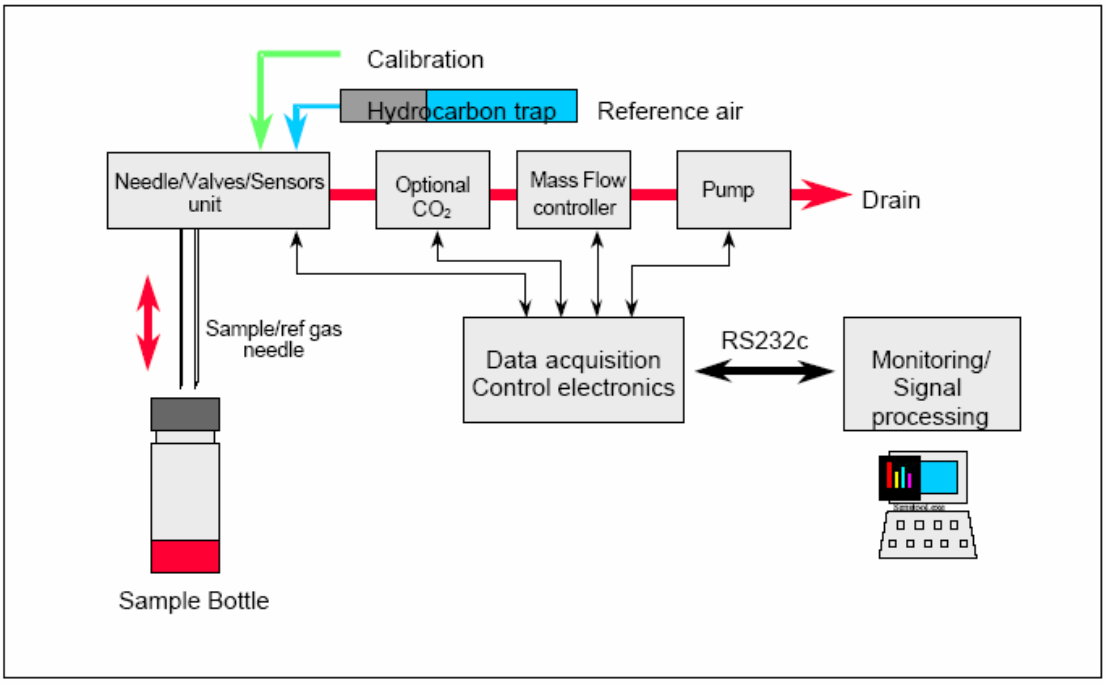



**Figure B.1:** NST system's operating principle (NST Senstool manual extras, 1999).

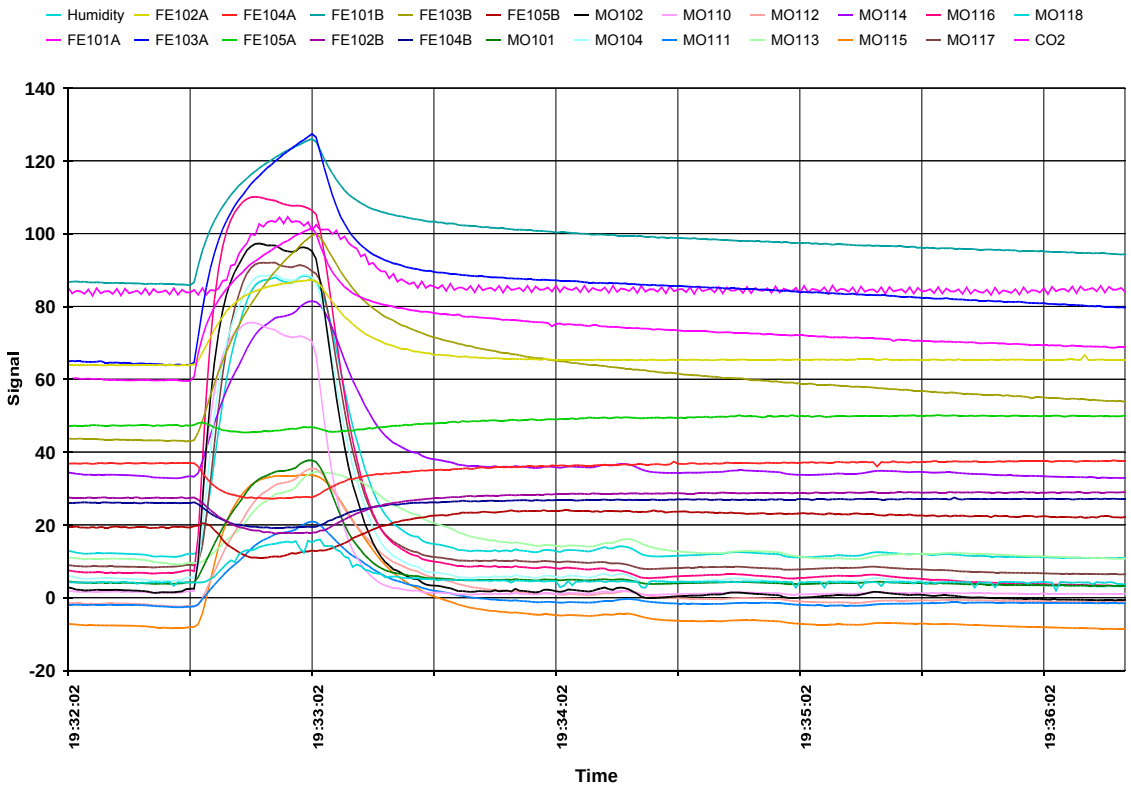



**Figure B.2:** The typical sensor responses to a particular sample.

## B.2 Testing sensors with standards

The sensors were tested over time for the effect of drift using certain standard controls such as 0.1 % acetone (1ml), 0.1% propan-1-ol (1ml), 0.05% propan-2-ol (1ml) and air. Figure B.3A depicts a PCA scores plot showing the clear separation between the different groups of controls (i.e. acetone, alcohols and air), but there appears to be signs of drift between the chemical compounds although the pattern of differentiation seems to be maintained. This drift over time is much clearer when PC1 and PC3 are observed (Figure B.3B). The data presented in the plots are from two different time periods (run approximately seven-eight months apart).

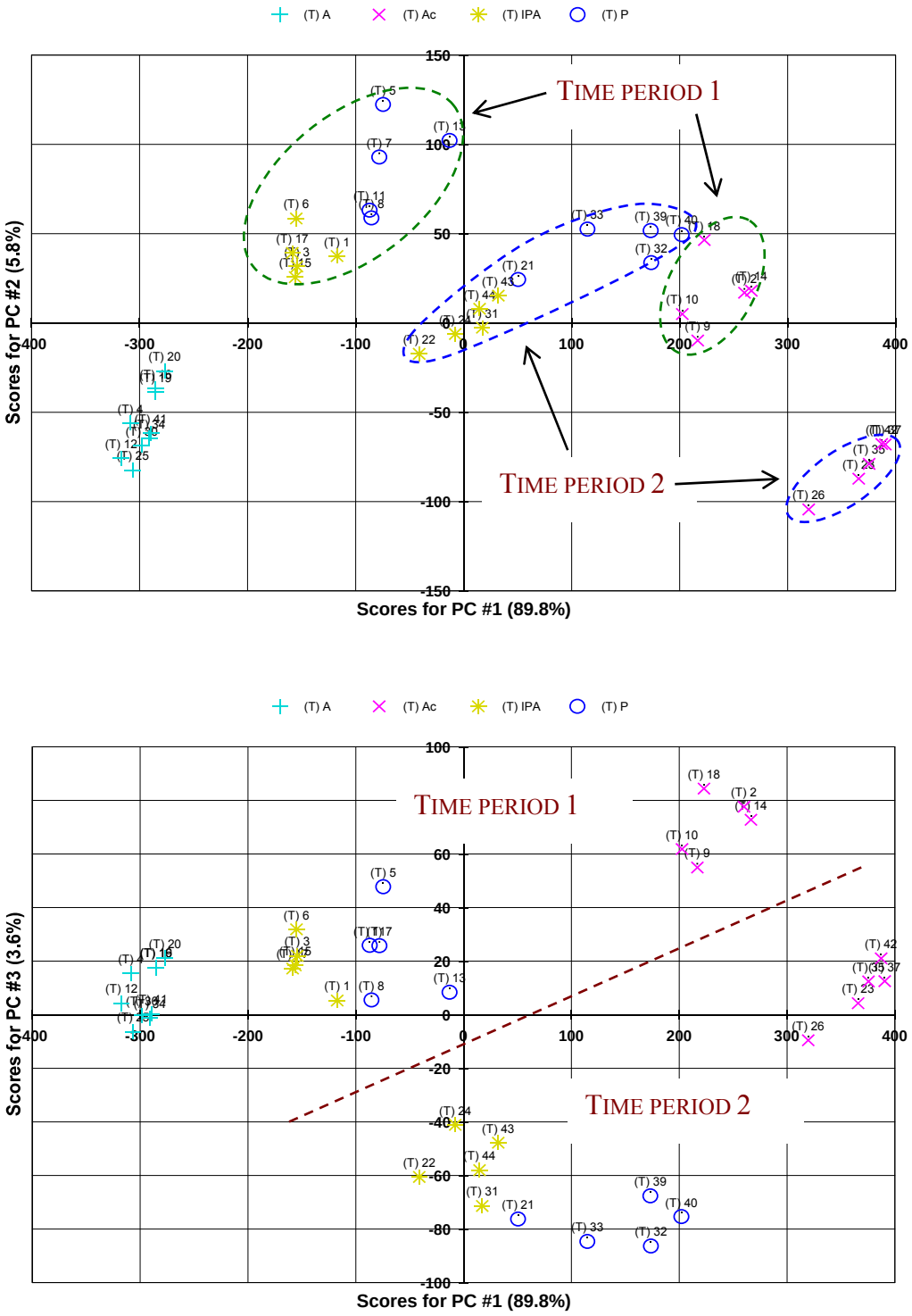



**Figure B.3:** PCA scores plots of certain standards depicting sensor drift over time for the chemical standards (A) which is much clearer when observing PC1 vs. PC3 (B). Samples 1-20 indicate time period 1 and those from 21 onwards indicate time period 2. (*Key:* A – reference air; Ac – acetone; IPA – propan-2-ol; P – propan-1-ol)

# *Appendix C – Probabilistic neural network (PNN) development*

PNNs are one of the variants of radial basis networks, which are two layered networks that work faster than conventional back propagation feed forward networks. However, they do require many more neurons. These networks are mainly used for classification purposes (Demuth & Beale, 2006), and thus used in the study to predict the classes of the fungal species.

In the PNN, the ultimate result is a vector that represents either a 1 for the correct or a 0 for an incorrect classification. The first layer calculates the distance between the weights and inputs, and uses a radial basis function (Eq. C.1) that creates an output vector with the help of the bias to indicate the closeness between the input and training sets. The second layer, a competitive layer, then uses the previous output to calculate the probabilities which then eventually results in a 1 or 0 using a compet function. Hence, it is because of the maximum probability of a specific class being true that the network classifies the inputs into those classes (Demuth & Beale, 2006).

$$Y_{radbas} = e^{-X^2} \qquad \ldots \textit{(Eqn C.1)}$$

where X is the product of the distances between the inputs and weights and the biases.

## *C.1 Network Architecture*

This two layered network also consists of an input layer made up of the same number of elements as there are sensors (in this case 5 or 18 or 24 sensors). The radial basis layer consists of as many nodes as there are samples or treatments in the training phase, whilst the final competitive layer consists of 5 neurons based on the fungal species. The topology of the network used in the *Trichophyton* species study is shown in Figure C.1.

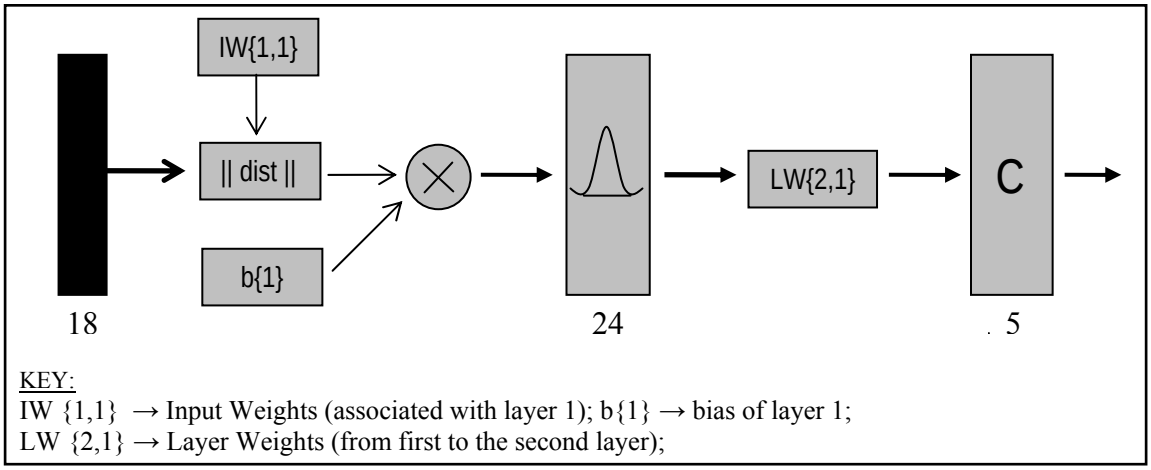



**Figure C.1:** Topology of the probabilistic neural network showing a single input comprising 18 elements, the layer with 24 neurons with the radbas transfer function and finally the compet transfer function producing the output.

### *C.2 Dataset Construction*

In case of any mathematical model that is built, it needs to be validated or tested on unseen data. Thus, the original dataset was split into training and test sets, each with their pair-wise combination of inputs (sensor responses) and targets (fungal classes). Due to the limited number of samples available, the datasets were generated using a process called *'leave one out cross validation'*; where one sample was excluded and the remaining were used for training the network. The sample that was omitted was used for testing the model. Thus, for the aforementioned scenario, 25 training and test sets were generated. Prior to feeding the training data into the neural network, it was normalised so that its interval was in the range of -1 to +1.

Subsequent to obtaining the result from the network, the accuracy of the classification was calculated and regression analysis performed.

### *C.3 Pseudocode*

- Load data
- For varying spread values (0 to 1 in steps of 0.1)
    - Spilt into training and test sets
    - Normalise the training sets
    - Create the network
    - Train and test the network
    - Save networks
- Calculate the classification accuracy
- Calculate the confusion matrix
- Perform regression analysis

# *Appendix D – Ethics approval*

## D.1 VAP study

NHS

**Gloucestershire Research Ethics Committee**
Gloucestershire Royal Hospital
Great Western Road
Gloucester
GL1 3NN

Tel: 01452 395726
Fax: 01452 395720

22 December 2005

Lee Humphreys
Clinical Research Fellow
Cranfield Postgraduate Medical School
Gloucestershire Royal Hospital
Great Western Road
Gloucester GL1 3NN

Dear Lee

**Study title:** **Diagnosis of Ventilator Associated Pneumonia: Tandem Breath Analyser versus Bronchoalveolar Lavage**
**REC reference:** **05/Q2005/119**
**Protocol number:** **Version 1**

**Amendment number:** **1**
**Amendment date:** **12 December 2005**

The above amendment was reviewed at the meeting of the Sub-Committee of the Research Ethics Committee held on 21 December 2005.

**Ethical opinion**

The members of the Committee present gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

**Approved documents**

The documents reviewed and approved at the meeting were:

- Notice of Substantial Amendment
- Protocol (Version 3, dated 12 December 2005)
- Retrospective Information Sheet (Version 2, dated 12 December 2005)
- Relative Information Sheet (Version 2, dated 12 December 2005)

**Membership of the Committee**

The members of the Ethics Committee who were present at the meeting are listed on the attached sheet.

An advisory committee to Avon, Gloucestershire and Wiltshire Strategic Health Authority

05/Q2005/119 Page 2

| | | |
|---|---|---|
| Participant Consent Form | 2 | 10 October 2005 |
| Response to Request for Further Information | | 07 December 2005 |
| CV for Catherine Kendall | | |
| Declaration of Conformity | | 10 October 2001 |
| Relative information Sheet | 2 | 01 December 2005 |
| Retrospective Information Sheet | 1 | 07 December 2005 |
| Assent Form | 2 | 21 November 2005 |

**Research governance approval**

The study should not commence at any NHS site until the local Principal Investigator has obtained final research governance approval from the R&D Department for the relevant NHS care organisation.

**Statement of compliance**

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

| | |
|---|---|
| **05/Q2005/119** | **Please quote this number on all correspondence** |

With the Committee's best wishes for the success of this project

Yours sincerely

**Alix Stevenson**
**Chair**

Email: sue.starck@glos.nhs.uk

*Enclosures:* *Standard approval conditions*
*Site approval form*

Copy to: Gloucestershire Hospitals NHS Foundation Trust
Trust HQ
1 College Lawn
Cheltenham
GL53 7AG

SF1 list of approved sites

An advisory committee to Avon, Gloucestershire and Wiltshire Strategic Health Authority

NHS

**Gloucestershire Research Ethics Committee**
Gloucestershire Royal Hospital
Great Western Road
Gloucester
GL1 3NN

Telephone: 01452 395726
Facsimile: 01452 395720

9 December 2005

Mr Martyn LEE Humphreys
Clinical Research Fellow
Gloucestershire Hospitals NHS Foundation Trust
Cranfield Postgraduate Medical School
Gloucestershire Royal Hospital
Great Western Road
Gloucester
GL1 3NN

Dear Mr Humphreys

**Full title of study:** **Diagnosis of Ventilator Associated Pneumonia: Tandem Breath Analyser versus Bronchoalveolar Lavage**

**REC reference number:** **05/Q2005/119**

Thank you for your letter of 7 December 2005, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

**Confirmation of ethical opinion**

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

**Ethical review of research sites**

The favourable opinion applies to the research sites listed on the attached form.

**Conditions of approval**

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

**Approved documents**

The final list of documents reviewed and approved by the Committee is as follows:

| *Document* | *Version* | *Date* |
|---|---|---|
| Application | | 10 October 2005 |
| Investigator CV | | |
| Protocol | 1 | 10 October 2005 |
| Peer Review | | 03 October 2005 |
| Participant Information Sheet | 1 | 10 October 2005 |

**Research governance approval**

All investigators and research collaborators in the NHS should notify the R&D Department for the relevant NHS care organisation of this amendment and check whether it affects research governance approval of the research.

**Statement of compliance**

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

| **05/Q2005/119:** | **Please quote this number on all correspondence** |
|---|---|

Yours sincerely

Hazel

**Hazel Gage**
**Committee Co-ordinator**

E-mail: hazel.gage@glos.nhs.uk

*Copy to:* *Dr Birgit Whitman, GHNHSFT*

*Enclosures* *List of names and professions of members who were present at the meeting and those who submitted written comments*

An advisory committee to Avon, Gloucestershire and Wiltshire Strategic Health Authority

## D.2 Upper GI Study

Gloucestershire Hospitals NHS
NHS Foundation Trust
1 College Lawn
Cheltenham
Gloucestershire, GL53 7AG
Tel: 08454 222860 Fax: 08454 223547
e-mail: sally.pearson@glos.nhs.uk

*Our Ref: SP/RF/05/Q2005/58*
*Your Ref:*

3rd November 2005

Mr M Lee Humphreys
Clinical Research Fellow
Biophotonics Research Group
Unit 7, Pullman Court
Gloucestershire Royal Hospital
Great Western Road
Gloucester GL1 3NN

Dear Lee

**Study No 05/Q2005/58 : Volatile Fingerprints and Diagnosis in the Upper Gastrointestinal Tract using the Tandem Breath Analyser**

Thank you for forwarding information on the above study. I can confirm the approval of Gloucestershire Hospitals NHS Foundation Trust for this study to proceed.

Your project will now be added to the Trust's Research Register which will identify the following:

- Principal Investigator: Lee Humphreys
- Sponsoring Organisation: Gloucestershire Hospitals NHS Foundation Trust
- Host Organisation: Gloucestershire Hospitals NHS Foundation Trust
- Type of Study: Local
- Proposed Completion Date: February 2007

It is important that your research complies with the Research Governance Framework. Enclosed for your information is a leaflet on research governance and a chart setting out the responsibilities of the principal investigator. During the course of your research I may need to contact you for further information in relation to your study and on completion of your study you will be expected to produce a summary of the project and an indication of how the results from the study will be disseminated.

I wish you every success with your project.

Yours sincerely

**Dr Sally Pearson**
**Director of Clinical Strategy**
**(Research Governance Lead)**

Enc

c.c. *Hazel Gage*

**Gloucestershire Research Ethics Committee**
Gloucestershire Royal Hospital
Great Western Road
Gloucester
GL1 3NN

Telephone: 01452 395726
Facsimile: 01452 395720

15 September 2005

Mr Martyn LEE Humphreys
Clinical Research Fellow
Gloucestershire Hospitals NHS Foundation Trust
Cranfield Postgraduate Medical School
Gloucestershire Royal Hospital
Great Western Road
Gloucester
GL1 3NN

Dear Lee

**Full title of study:** **Volatile Fingerprints and Diagnosis in the Upper Gastrointestinal Tract using the Tandem Breath Analyser.**

**REC reference number:** **05/Q2005/58**

Thank you for your letter of email 8 September 2005, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

**Confirmation of ethical opinion**

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

The favourable opinion applies to the research sites listed on the attached form.

**Conditions of approval**

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

**Approved documents**

The final list of documents reviewed and approved by the Committee is as follows:

| Document | Version | Date |
|---|---|---|
| Application | 3 | 03 June 2005 |
| Investigator CV | | (None Specified) |
| Protocol | 1 | 18 May 2005 |
| Participant Information Sheet | 3 | 08 September 2005 |
| Participant Consent Form | 2 | 12 July 2005 |
| Patient Pre-Information Sheet | 2 | 12 July 2005 |
| Email from Daniella Rogers, MHRA | | 23 June 2005 |
| Declaration of Conformity | | 10 August 2005 |
| CV for Catherine Kendall | | (None Specified) |

**Gloucestershire Research Ethics Committee**

**LIST OF SITES WITH A FAVOURABLE ETHICAL OPINION**

*For all studies requiring site-specific assessment, this form is issued by the main REC to the Chief Investigator and sponsor with the favourable opinion letter and following subsequent notifications from site assessors. For issue 2 onwards, all sites with a favourable opinion are listed, adding the new sites approved.*

| **REC reference number:** | 05/Q2005/58 | **Issue number:** | 1 | **Date of issue:** | 15 September 2005 |
|---|---|---|---|---|---|
| **Chief Investigator:** | Mr Martyn LEE Humphreys | | | | |
| **Full title of study:** | Volatile Fingerprints and Diagnosis in the Upper Gastrointestinal Tract using the Tandem Breath Analyser. | | | | |

*This study was given a favourable ethical opinion by Gloucestershire Research Ethics Committee on 15 September 2005. The favourable opinion is extended to each of the sites listed below. The research may commence at each NHS site when management approval from the relevant NHS care organisation has been confirmed.*

| Principal Investigator | Post | Research Site | Site assessor | Date of favourable opinion for this site | Notes (1) |
|---|---|---|---|---|---|
| Mr Martyn LEE Humphreys | Clinical Research Fellow | Gloucestershire Hospitals NHS Foundation Trust | Gloucestershire Research Ethics Committee | 15/09/2005 | |

Approved by the Chair on behalf of the REC:

Hazel Grey .....(Signature of Chair/Administrator)

(delete as applicable)

HAZEL GRAY .....(Name)

(1) *The notes column may be used by the main REC to record the early closure or withdrawal of a site (where notified by the Chief Investigator or sponsor), the suspension of termination of the favourable opinion for an individual site, or any other relevant development. The date should be recorded.*

An advisory committee to Avon, Gloucestershire and Wiltshire Strategic Health Authority

# *Appendix E – Publications, Oral presentations and Posters*

## E.1 Papers

**Sahgal, N.**, Monk, B., Wasil, M. & Magan, N. (2006). Trichophyton species: use of volatile fingerprints for rapid identification and discrimination. *Br J Dermatol* 155(6):1209-1216. (doi:10.1111/j.1365-2133.2006.07549.x)

**Sahgal, N.**, Needham. R., Cabañes, F. J. & Magan, N. (2007). Potential for detection and discrimination between mycotoxigenic and non-toxigenic spoilage moulds using volatile production patterns: a review. *Food Additives and Contaminants* 24(10):1161-1168. (doi:10.1080/02652030701519096)

**Sahgal, N.** & Magan, M. (2008). Fungal volatile fingerprints: discrimination between dermatophyte species and strains by means of an electronic nose. *Sensors & Actuators B: Chemical* 131(1): 117-120. (doi:10.1016/j.snb.2007.12.019)

**Sahgal, N.**, Turner, C. & Magan, N. Identification of potential volatile markers generated by common dermatophyte species using mass spectrometric techniques. (Submitted to *Br J Dermatol*)

## E.2 Book Chapters

Magan, N. & **Sahgal, N.** (2007). Electronic sensing: food and feed applications. In*: Rapid methods for food and feed quality determination*, pp 15-28. Edited by: A.van Amerongen, D. Barug & M. Lauwaars: Wageningen Academic Publishers.

Magan, N. & **Sahgal, N.** (2007). Electronic nose for quality and safety control. In *Advances in Food Diagnostics*, pp. 119-129. Edited by L. Nollet & F. Toldra: Blackwell Publishing.

## E.3 Oral presentations

**Sahgal, N.** & Magan, M. Fungal volatile fingerprints: discrimination between dermatophyte species and strains by means of an electronic nose. Presented at the *12th International Symposium on Olfaction & Electronic Nose*, May 2007, St. Petersburg, Russia.

**Sahgal, N.** & Magan, M. Fungal Volatile Fingerprints - species and strain discrimination of dermatophytes. Presented at *the Annual British Mycological Society Meeting*, September 2007, Manchester, UK.

## E.4 Posters

**Sahgal, N.**, Monk, B., Wasil, M. & Magan, N. Use of electronic nose for early detection and discrimination between dermatophytes. *11th International Symposium on Olfaction and Electronic Nose*, April 2005, Barcelona, Spain.

**Sahgal, N.**, Monk, B., Wasil, M. & Magan, N. Potential of electronic nose technology for early detection and discrimination between dermatophytes. *8th International Mycological Congress*, August 2006, Cairns, Australia.

**Sahgal, N.**, Monk, B., Wasil, M. & Magan, N. (2007). Fungal volatile fingerprints and machine learning: potential of discriminating and classifying dermatophyte species. *BioSysBio: Systems Biology, Bioinformatics and Synthetic Biology*, January 2007, Manchester, U.K *[BMC Sys Biol* 1(Suppl 1):P37*]*.

**Humphreys, M. L.**, Orme, R., Smith, S., **Sahgal, N.** & other authors. Electronic Nose Analysis of Bronchoalveolar Lavage Fluid for the Diagnosis of Ventilator-associated Pneumonia. *36th Critical Care Congress*, February 2007, Orlando, Florida.

**Humphreys, M. L.**, Orme, R., **Sahgal, N.**, Kendall, C., Magan, N. & Stone, N. Electronic Nose Analysis of Bronchoalveolar Lavage Fluid for the Diagnosis of Ventilator-associated Pneumonia. *Intensive Care Society's (ICS) State of the art meeting*, December 2007, London, UK.